Relationship Between Tumor Necrosis Factor-α and β-Adrenergic Receptors in C6 Glioma Cells

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Abstract

Damage to the central nervous system (CNS) elicits complex responses, which subsequently leads to glial scar formation, and this process has been shown to impede neuron regeneration. Among the responses, it was observed that the level of tumor necrosis factor-α (TNF-α) was greatly elevated at the site of injury, and that inhibitors of TNF-α gene expression significantly improved the outcome of injury suggesting that TNF-α mediates pathological events following brain injury. Accumulated evidence showed that astrocytes, the most abundant cell type in the CNS, were activated by cytokines, including TNF-α, then underwent proliferation and astrogliosis, both processes are closely related to glial scar formation. Recently, it was also found that β-adrenergic blockade reduced astrogliosis suggesting that β-adrenergic mechanism was closely related to glial scar formation. However, the relationship between the TNF-α and β-adrenergic-mediated events remains unclear. The aims of this project are (i) to investigate whether there is a relationship between TNF-α receptors (TNF-R) and β-AR expression, and (ii) the messenger mediating these two processes.

Results of the present study indicated that TNF-α induced the expression of TNF-R2 and β1- and β2-ARs, but that of TNF-R1 was unaffected. The activation of

these receptors has been shown to mediate C6 cell proliferation. The induction of β-ARs was stimulated by phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, while Ro-31-8220 (Ro31), a potent PKC inhibitor, suppressed TNF-α-induced stimulation. In addition, we observed that TNF-α, PMA and isoproterenol (an β-adrenergic agonist) selectively induced the expression of TNF-R2. As both TNF-α and isoproterenol have previously been shown to stimulate proliferation, these data indicated that TNF-α–induced proliferation in C6 glioma cells via the induction of TNF-R2 and β-AR genes, and this induction was mediated through PKC. Moreover, we found that β-agonists stimulated, but β-antagonists suppressed TNF-α and TNF-α-induced TNF-R2 expression. This demonstrated that there was an interaction between TNF-α and β-adrenergic mechanisms in C6 cells.

Our results showed that TNF-α, PMA and isoproterenol induced the transcription factor, nuclear factor-κB (NF-κB) and its target gene, manganese superoxide dismutase (MnSOD) expression, in C6 cells. On the other hand, propranolol attenuated the TNF-α-induced NF-κB and MnSOD expression in C6 cells. These indicated that NF-κB and MnSOD expression induced by TNF-α was mediated via PKC and β-adrenergic systems in C6 cells.

Taken together, this study suggests that regulation of PKC, NF-κB and MnSOD should be beneficial therapeutic means, in addition to suppression of TNF-α and β-

adrenergic mechanisms, in treating brain injury.

摘要

中樞神經系統(CNS)的損壞會引起複雜的反應，隨後致使爲神經膠質疤之形成，從而阻礙神經元的再生 。據觀察所得，發現腫瘤壞死因子(TNF-α)的數量於腦部受損之處大大增加，而 TNF-α之基因表達抑制劑明顯地促進了腦部損傷後的復原。這顯示出 TNF-α可能是導致腦部受損後一連串病變的媒體。再者，有很多的証據顯示出 CNS 數目最多的星形膠質細胞，會被多種不同細胞激素，例如 TNF-α所刺激，而產生神經膠質過多症，這是形成神經膠質疤的一過程 。最近的研究報告中指出β-腎上腺素的抑制減少神經膠質過多症 。這表示出β-腎上腺素的機制跟神經膠質疤之形成有密切關係。但是，TNF-α與β-腎上腺素的機制之相互關係仍然不清楚。這實驗之目的是: (i) 研究 TNF-α跟β-AR 的表達過程中是否有關係，及(ii) 連接此兩機制的信使。

我們的實驗結果証實 TNF-α能引起 TNF-α受體二型(TNF-R2), β-腎上腺素受體一型(β1-AR)及二型(β2-AR)之表達，但對 TNF-α受體一型(TNF-R1)卻沒有影響 。這些受體皆能使 C6 細胞增生。而蛋白激霉丙型(PKC)的激活劑 phorbol-12-myristate-13-acetate (PMA)能刺激 β-AR 的表達，而 PKC 的抑制劑 Ro-31-8220(Ro31)可壓止 TNF-α所引發的反應 。除此之外，我們發現 TNF-α, PMA 和 β-AR 的激活劑(isoproterenol)都可選擇性地誘發 TNF-R2 之表達 。我們早前的實驗結果發現 TNF-α和 isoproterenol 皆能刺激 C6 神經膠質瘤細胞之增殖 。這些可表明 TNF-α所誘發的 C6 神經膠質瘤細胞的增殖，是透過 TNF-α跟β-AR 基因表達而產生，而 PKC 爲一重要信使 。此外，我們亦發現β-AR 的激活劑誘發 TNF-α和 TNF-R2 之表達，而其抑制劑壓止它們的表達 。這証明了β-腎上腺素及 TNF-α兩機制有相互作用 。

我們的實驗結果也顯示 TNF-α，PMA 和 isoproterenol 可誘發核因子-κB (NF-κB)和它的目標基因錳超氧物歧化霉(MnSOD)在 C6 細胞中之表達 。另一方

面，propranolol 亦可減弱 TNF-α於 C6 細胞中所誘發 NF-κB 跟 MnSOD 的表達。這些結果都証明了 TNF-α在 C6 細胞中可透過 PKC 和β-腎上腺素系統而對 NF-κB 跟 MnSOD 的表達引起作用。

總括來說，本實驗顯示除抑制腦部 TNF-α及β-腎上腺素機制外，控制 PKC、NF-κB 及 MnSOD，也是良好醫治腦部損傷的方法。

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List of Abbreviations

AA	arachidonic acid
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
ATP	adenosine triphosphate
β-actin	beta-actin
β-ARs	beta-adrenergic receptors
BBB	blood-brain barrier
bp	base pair
cAMP	cyclic adenosine-3',5'-monophosphate
cDNA	complementary deoxyribonucleic acid
CM	cerebral malaria
CNS	central nervous system
CSF	cerebrospinal fluid
Cu-ZnSOD	copper-zinc superoxide dismutase
DAG	diacylglycerol
dbcAMP	N^6 -2'-dibutyryl cyclic adenosine-3',5'-monophosphate
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
EDTA	ethylenediaminetetraacetic acid disodium salt
FBS	fetal bovine serum
Fig.	figure
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase

H_2O_2	hydrogen peroxide
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HIV	human immunodeficiency virus
HSP27	heat shock protein-27
ICAM	intracellular adhesion molecule
IFN	interferon
IL-1	interleukin-1
IL-4	interleukin-4
IL-6	interleukin-6
ISO	isoproterenol
kDa	kilodalton
LPS	lipopolysaccharide
MAP kinase	mitogen-acivated protein kinase
MHC	major histocompatibility complex
MnSOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
NE	norepinephrine
NF-κB	nuclear factor-kappa B
NO	nitric oxide
OD	optical density
p50	p50 subunit of nuclear factor-kappa B
p65	p65 subunit of nuclear factor-kappa B
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PKA	protein kinase A

PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
RNA	ribonucleic acid
Ro-31	3-{1-[3(amidinothio)propyl]-3-indolyl}-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione methanesulfonate
ROSs	reactive oxygen species
RPMI Medium	Rosewell Park Memorial Institute medium
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
sHSPs	small heat shock proteins
SMase	sphingomyelinase
SOD	superoxide dismutase
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
TK	tyrosine kinase
TNF-α	tumor necrosis factor-alpha
TNF-R	tumor necrosis factor receptor
Tris	Tris (hydroxymethyl) aminomethane

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1 INTRODUCTION

Tumor necrosis factor-α (TNF-α), a cytokine, is a pleiotropic polypeptide, which plays a significant role in immune and inflammatory activities in many organs, including the brain. For example, TNF-α has been found to be increased in the brain after mechanical trauma and has been suggested to serve as an important mediator of inflammation and microvascular injury associated with trauma (Kamei *et al.*, 2000). Also, an increase in TNF-α mRNA expression has been reported following traumatic brain injury (Fan *et al.*, 1996). These suggest that TNF-α plays important roles in brain injury.

Apart from the increase in TNF-α, it has been shown that β-adrenergic receptors (β-ARs) was activated following brain injury (Hodges-Salova *et al.*, 1996), and they play important roles mediating injury-induced astrogliosis and cell proliferation in the central nervous system (CNS) (Griffith & Sutin, 1996). However, the relationship between increased TNF-α and β-AR activation remains to be established. Recently, it was reported that TNF-α selectively induced the expression of TNF receptor 2 (TNF-R2) in C6 glioma cells (Huang *et al.*, 1998), a receptor responsible for proliferation (Bluethmann, 1998) and β-ARs in C6 glioma cells (Lung, 1999).

The above reports suggest that TNF-α might induce astrogliosis and scar formation through β-ARs. This study is to further characterize the relationship between β-ARs upon TNF-α treatment and the signaling pathway mediating these events in C6 glioma cells.

1.1 What are the general functions of cytokines?

Communication between cells in the immune and hematopoietic systems is mediated by soluble factors called interleukins (IL) or cytokines.

Cytokines are proteins produced by cells in response to a variety of inducing stimuli. They are secreted by their producer cells to influence the behaviour of their target cells. Cytokines exert their effects by binding to their specific receptors on the surfaces of their target cells. The resulting effects inside the target cell are brought about by signal transduction across the plasma membrane (Clemens, 1991) as diagrammatically illustrated in Fig. 1.

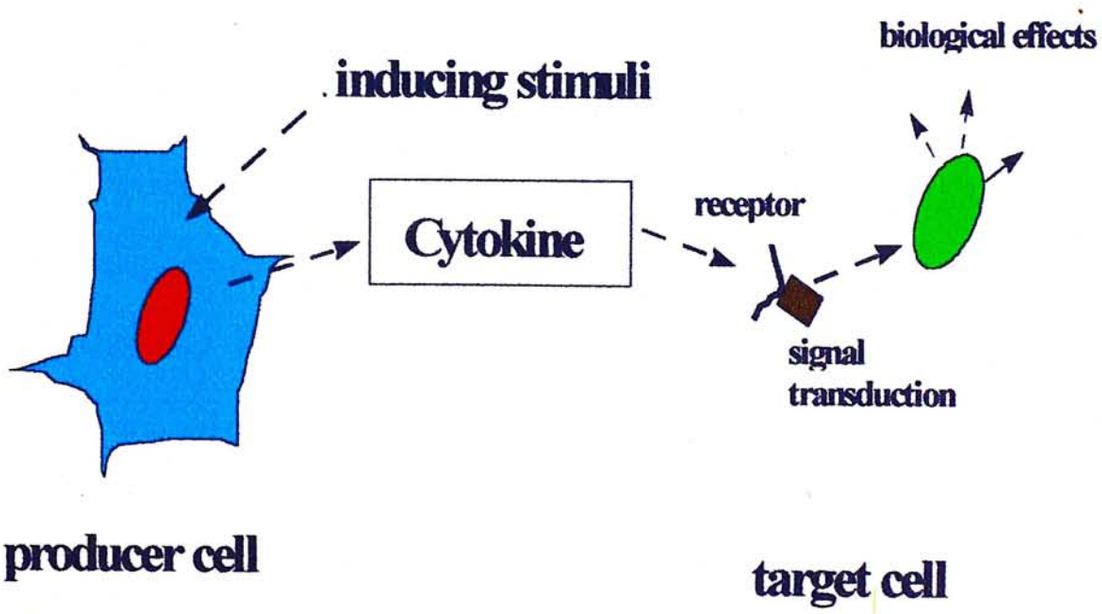

Fig. 1 Action of cytokines (abridge from Clemens, 1991).

Most of the physiological roles of the cytokines are related specifically to the requirements of multicellular organisms, and are concerned either with co-ordination of processes between different cell types, or with the response to environmental stresses. It is not surprising, therefore, that many diseases involving disruption of these processes are associated with altered regulation of cytokine production and action (Clemens, 1991).

Normal cells will generally proliferate in response to specific signals, such as cytokines. In addition, cytokines are also concerned with controlling cell growth and differentiation (Clemens, 1991).

Moreover, there are numerous ways in which the immune system responds to invasion by foreign organisms or other antigenic agents, and one of the more interesting means is the production of cytokines. Since cytokines are produced under appropriate circumstances, cells normally do not possess stores of cytokine molecules waiting to be secreted in response to a stimulus. Nevertheless, cytokines are produced rapidly by *de-nove* synthesis in response to specific stimuli (Clemens, 1991).

Previously, cytokines were believed to be closely associated with the immune system only, however, recently several cytokines and their respective receptors have been identified in many organs, e.g., the brain, and cytokines were found in cerebrospinal fluid. These observations suggest that cytokines play a key role in mediating communication between the nervous and immune systems. One of cytokines in the CNS that has drawn much attention these days is TNF-α (Kamei *et al.*, 2000; Wong *et al.*, 1996). This is because this cytokine is closely related to brain injury (Fan *et al.*, 1996; Kamei *et al.*, 2000).

1.2 What is TNF-α?

TNF-α is a 17kD protein produced mainly by activated macrophages in response to wide variety of stimuli including mitogens, cytokines, bacteria, viruses and parasites. In addition, TNF-α has been shown to take part in altering vascular endothelial cell functions during inflammation (Zhu *et al.*, 2000). These suggest that TNF-α is an inflammatory cytokine. TNF-α is also known to stimulate other cell types, such as astrocytes, to produce cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), colony stimulating factors and TNF-α itself (Balkwill, 1995). This further suggests the importance of TNF-α in brain function.

TNF-α exists in its biologically active and physiological form as a homotrimer with a molecular mass of 52kDa. Well-diffracting crystals of TNF-α had allowed the three-dimensional structure to be determined as shown in Fig. 2 (Balkwill, 1995). The shape of the TNF-α homotrimer has the appearance of a triangular cone, or bell, in which each of the three subunits has a typical jelly roll-β structure and the three subunits are arranged edge to face (Fig. 2).

In the brain, TNF-α derived from cerebral or blood-derived cells, may exert a positive regulation on platelet-derived growth factor (PDGF) synthesis in human astrocytes (Silberstein *et al.*, 1996). PDGF is a pleiotropic cytokine that acts on many different cell types (e.g., fibroblasts; smooth muscle, endothelial and neural cells), and is able to stimulate a variety of cellular responses, including proliferation, chemotaxis, actin reorganization, and Ca^{2+} mobilisation (Heldin & Westermark, 1990; Raines *et al.*, 1990). These reports emphasized the importance of TNF-α (and other cytokines) in normal functions and pathological states of the nervous system.

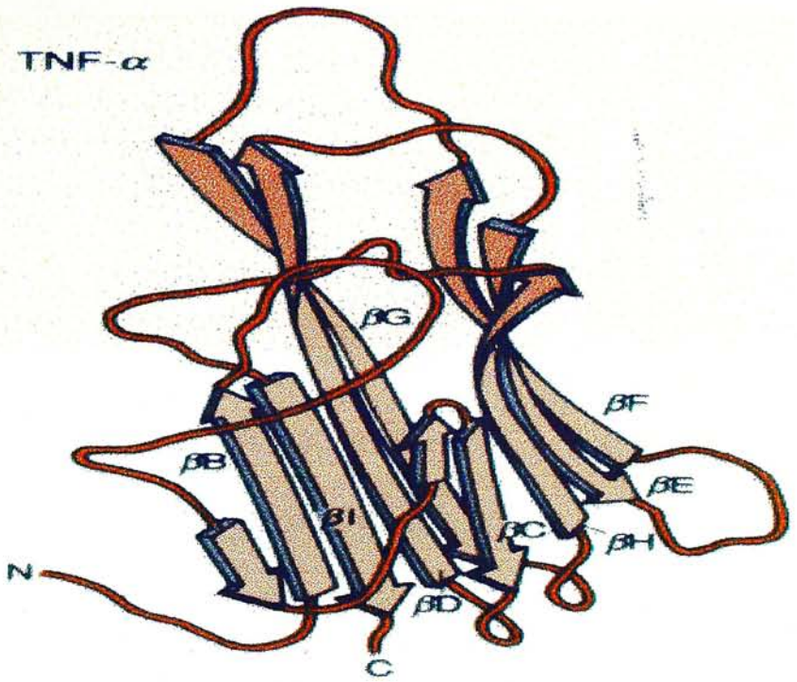

Fig.2 The TNF-α molecule. Space-filling model of the TNF-α homotrimer binding to three p55 TNF receptors (TNF-R1) (abridge from Balkwill, 1995).

1.3 Actions of TNF-α

TNF-α production and secretion can be induced in macrophages in response to bacterial lipopolysaccharide (LPS) (Nakamura *et al.*, 1999). In the CNS, TNF-α has been shown to activate the endothelium for leukocyte adherence and procoagulation activity (by increasing von Willebrand factor and platelet activating factor) that can exacerbate ischemic damage in the brain (Pober *et al.*, 1990). Indeed, increased TNF-α in the brain and blood in response to LPS appears to contribute to increased brain stem thrombosis and hemorrhage (Hallenbeck *et al.*, 1988; Hallenbeck *et al.*, 1991; Siren *et al.*, 1992) and can contribute to increased stroke sensitivity/risk in hypertensive rats (Barone *et al.*, 1992). TNF-α is known to stimulate other cell types to produce cytokines, including IL-1, IL-6, colony stimulating factor, and TNF-α itself (Aggarwal and Vilcek, 1992). Moreover, TNF-α plays a pivotal role in inflammatory processes

(Strieter *et al.*, 1993). It activates neutrophils (Shalaby *et al.*, 1985), increases leukocyte-endothelial cell adhesion molecule expression (Pober *et al.*, 1990), and increases leukocyte adherence to blood vessels and their subsequent infiltration into the brain (Liu *et al.*, 1994).

TNF-α has been shown to cause oligodendrocytic damage, suggesting an important role of TNF-α in demyelination observed in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (Robbins *et al.*, 1987; Selmaj and Raine, 1988; Hofman *et al.*, 1989). In primary astrocytes, TNF-α increases class I major histocompatibility complex (MHC) and intercellular adhesion molecule-1 (ICAM-1) expression, and enhances class II MHC expression induced by interferon-γ (IFN-γ) or virus (Frohman *et al.*, 1989; Massa *et al.*, 1987). These indicate that astrocytes can function as antigen presenting cells (APCs) within CNS. In peripheral tissues, such as pancreatic cells, TNF-α has been shown to induce insulin secretion (Southern *et al.*, 1990). These illustrate the diverse actions of TNF-α in our body.

1.4 General functions of TNF-α in astrocytes

In the brain, TNF-α is secreted by astrocytes, microglia and some neurons. Some reports indicate that the primary role for the macrophage-derived cytokine TNF-α acts as a growth promoting factor for the astrocytes, such as in the induction of astrocyte proliferation (Selmaj *et al.*, 1990). TNF-α has been recently shown to influence neuronal progenitor cell proliferation and differentiation (Wong *et al.*, 1996). However, very little is known about the TNF-α postreceptor signal transduction mechanism that leads to either cell growth or differentiation.

Moreover, TNF-α has found to be an effector molecule of central importance to the pathogenesis of many infectious, inflammatory and autoimmune disorders (Vassalli, 1992). In addition to its well-studied activities in the peripheral immune system, TNF-α has also been reported to play a key pathogenic role in a range of human neuroinflammatory diseases, such as MS (Selmaj *et al.*, 1991), bacterial meningitis (Leist *et al.*, 1988), and cerebral malaria (Grau *et al.*, 1989, Akassoglou *et al.*, 1997). TNF-α has also been implied in the pathogenesis of various immune mediated processes, and is the key mediator in septic shock (Tracey *et al.*, 1991). In the last decade, investigations also focused on the role of cytokines in traumatic brain injury (Goodman *et al.* 1990; Morganti-Kossmann *et al.*, 1992; Morganti-Kossmann & Kossmann, 1995; Morganti-Kossman *et al.*, 1997; Ross *et al.*, 1994; Shohami *et al.*, 1999). The important role of TNF-α in brain injury is further supported by findings that TNF-α mRNA expression so as its protein were increased in the brain after brain injury (Fan *et al.*, 1996). These investigators found that significant increase in TNF-α mRNA expression was observed one hour after brain injury. So, the post-traumatic alteration in the gene expression of TNF-α may play an important role in both the acute and regenerative responses to CNS trauma.

Little is known about the molecular mechanism responsible for the multiple biological activities of TNF-α in CNS trauma. However, the first step, as is the case for most polypeptide hormones, is the binding to specific cell-surface receptors which then activates a variety of biological signals that lead to different effects of the target cell.

1.5 TNF-α receptors (TNF-Rs)

The fact that TNF-α receptors (TNF-Rs) are involved in mediating the actions of TNF-α is readily supported by the observation that TNF-α binding activity to cell-surface receptors correlate with cell stimulation (Camussi *et al.*, 1991). At present, both high-affinity (K_d = 0.1 pM) and low-affinity (K_d = 0.1 nM) binding sites had been identified (Smith *et al.*, 1989) having a molecular mass of approximately 300kDa, possibly composed of dissimilar subunits (Creasy *et al.*, 1987). These types of the TNF-R cDNA have been cloned, indicating the presence of two species of TNF-α binding proteins (Schall *et al.*, 1990; Loetscher *et al.*, 1990; Smith & Baglioni, 1989), named type 1 (TNF-R1) (Loetscher *et al.*, 1990; Kohno *et al.*, 1990) and type 2 (TNF-R2) (Smith *et al.*, 1989; Kohno *et al.*, 1990) receptor (Fig. 3).

TNF-Rs exist in both bound and free forms. The soluble form of TNF-α receptor has been detected in urines of healthy subjects (Engelmann *et al.*, 1990) and in the sera of cancer patients (Schall *et al.*, 1990) and is probably a ' shed ' form of cell receptor. This soluble receptor may compete with the cell-surface receptor in the binding of TNF-α, thus acting as a physiological inhibitor or competitor.

Comparison between the human TNF-R1 and TNF-R2 showed that TNF-R1 of the rat is most conserved in the extracellular domain (70% identity) while the TNF-R2 is most conserved in the intracellular domain (73% identity). The weaker homology of the extracellular domain may help to explain the species specificity of TNF-R2 (Lewis *et al.*, 1991). However, there is a complete absence of homology between the intracellular domains of the two TNF-Rs, suggesting that they utilize distinct signaling pathways (Lewis *et al.*, 1991).

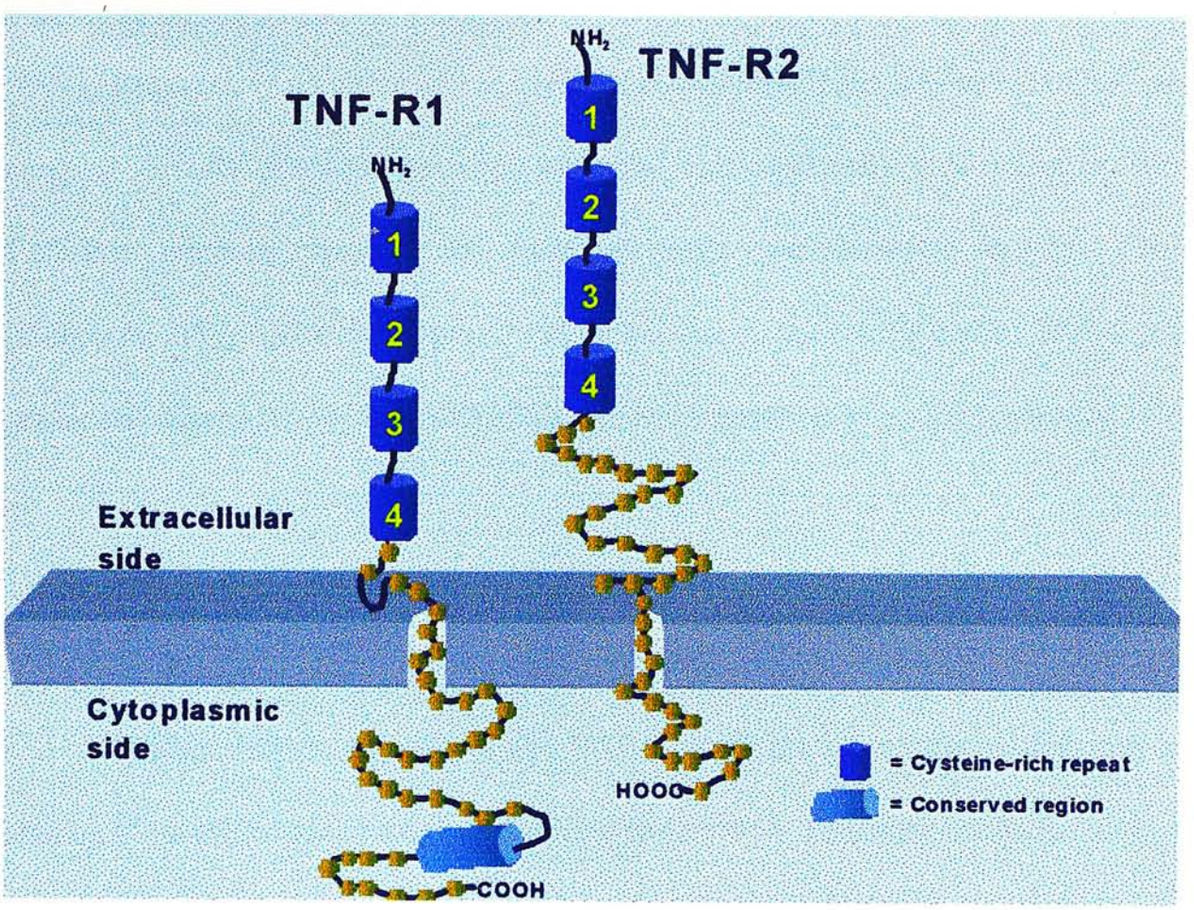

Fig. 3 Structures of TNF-α receptors, TNF-R1 and TNF-R2 (abridge from Foxwell *et al.*, 1992).

Both TNF-Rs are active in signal transduction and that there is redundancy in the function of the two receptors. TNF-R1 is the biologically relevant TNF receptor and that the binding of TNF-α to TNF-R2 is not sufficient to initiate TNF responses. For example, Tartaglia *et al.* (1991) proposed that TNF-R1 mediates a large number of diverse TNF-α activities, such as cytotoxicity, manganese superoxide dismutase (MnSOD) and nuclear factor-kappa B (NF-κB) induction, while TNF-R2 signals for the proliferation of primary thymocytes and T cells. At low concentrations of TNF-α,

TNF-α would preferentially bind TNF-R2 since the affinity of TNF-α for TNF-R2 is higher than for TNF-R1 (Bluethamann, 1998).

Most cell types express both TNF-R1 and TNF-R2, but expression of one type of receptor usually predominates. For example, epithelial cells predominantly express TNF-R1, whereas myeloid and lymphoid cells predominantly express TNF-R2 (Hohmann *et al.*, 1989; Ware *et al.*, 1991). So, the response of a cell to TNF-α is thus profoundly shaped by the type of TNF-R it predominantly expresses (Dopp *et al.*, 1997).

1.6 Second messengers induced by TNF-α

The mechanism through which TNF-α mediates its numerous activities after binding to its cell-surface receptors is poorly understood. This is probably due to the fact that the intracellular domains of the two TNF-Rs have no sequence homology to any other receptors (except for TNF-R1 and Fas antigen) nor to any known catalytic domains of protein kinases (Barbara *et al.*, 1996).

Some studies showed that when TNF-α binds to TNF-R1, diacylglycerol (DAG) is rapidly produced from membrane phospholipids by the activation of a phospholipase C (PLC) which then activates two signaling enzymes, a calcium-independent protein kinase C (PKC) and acidic sphingomyelinase (SMase) (Barbara *et al.*, 1996). SMase facilitates the breakdown of sphingomyelin to ceramide, a second messenger known to stimulate a number of cellular responses, including TNF-α-stimulated apoptosis (Obeid *et al.*, 1993; Hannun, 1994). These authors found that following TNF-α activation, SMase hydrolysed membrane sphingomyelin to ceramide resulting in the activation of a ceramide-activated protein kinase which is capable of phosphorylating a mitogen

activated protein (MAP) kinase. The activation of MAP kinase and PKC has been shown to be relevant to the functional status of phospholipase A_2 (PLA_2). PLA_2 is essential for the release of arachidonic acid (AA) from membrane phospholipids resulting in leukotriene and prostaglandin formation, and is necessary for the formation of platelet-activating factor which can increase neutrophil transmigration (Kuijpers *et al.*, 1992).

Another pathway involves the induction of a transcription factor which binds to κB-like enhancers, and this mechansim is thought to involve PKC (Barbara *et al.*, 1996), but there is also evidence that other kinases or signaling pathways may also particulate in the TNF-α signal transduction (Zhang *et al.*, 2000).

1.7 Glial Cells

As glial cell is one of the major sources of TNF-α in injured CNS, so the characteristics, properties and functions of glial cells are briefly discussed follow. The first account of neuroglia was cited by Dutrochet in 1824, who noticed the existence in the CNS of non-neuronal components made up of spindle-shaped cells which were morphologically distinct from neurons. These cells were considered as a form of connective tissue within the CNS, and were called 'neuroglia', which means nerve glue. There are three classes of nonneuronal cells which make up most of the glial population: astrocytes, oligodendrocytes, and brain macrophages, also called microglia (Benveniste, 1992). The basic properties of oligodendrocytes, microglia and astrocytes are briefly described below.

1.7.1 Oligodendroglia

Oligodendrocytes, like astrocytes, arise from neuroepithelial cells. Oligodendrocytes arise from the 0-2A progenitor cells, which are detected at embryonic day 21 (in the rat) (Raff *et al.*, 1983). These progenitor cells can differentiate into either fibrous astrocytes or oligodendrocytes, depending on environmental influences. In rats, most oligodendrocytes are generated in the period of 7-17 days postnatally, with the peak occurring at day 14 (Benveniste, 1992). The most commonly used marker for oligodendrocyte identification is galactocerebroside, the major glycolipid of myelin. In the setting of brain injury, oligodendrocytes are usually viewed as victims, not a defense system.

The function of the oligodendrocyte is myelin formation in the CNS. Myelin wraps around axons, acts as insulation for nerve fibers, and allows for efficient nerve impulse conduction (Morell *et al.*, 1980). Cytoplasmic projections extend from the oligodendrocyte cell body to wrap around nerve fibers in a spiral fashion. The oligodendrocyte is capable of producing many intermodes of myelin simultaneously; in rat optic nerve, a single oligodendrocyte can myelinate up to 50 separate axons (Benveniste, 1992).

1.7.2 Brain Macrophages (Microglia)

Virtually all body tissues contain cells competent to act as macrophages, and are able to recruit bloodborne macrophages. In the brain tissue, a cell population termed 'microglia' has the capacity to carry out phagocytosis after injury. All known phenotypic

markers for microglia are shared with other cell types, thus, there are no unique microglia-specific antigens (Benveniste, 1992). However, microglia can be identified by a number of cell surface antigens which include: immunoglobulin Fc receptors (Perry *et al.*, 1985), type 3 complement receptors (Perry *et al.*, 1985; Giulian & Baker, 1986) and β_2-integrins (Aliyama & McGeer, 1990). They can also be identified by the presence of nonspecific esterase (Suckling *et al.*, 1983).

The major subtypes of microglia include ramified, ameboid and perivascular microglia. Ramified microglia appear as highly branched small cells, with branching occurring in all planes. The branching of microglia cell processes is often found around neurons, suggesting that there may be a functional significance to this physical association. Perivascular microglia are found in the perivascular space, and are thought to be more closely related to monocytes than to ramified microglia. They do not have the extensive branched appearance of ramified microglia, and their cytoplasm often contains cytoplasmic vacuoles with lipid material. Ameboid microglia have a similar morphology as perivascular microglia, and can be unipolar or bipolar. In inflammatory conditions, it is the ameboid microglia that becomes activated and proliferates, ultimately forming microglial nodules as seen in patients with viral encephalitis and acquired immunodeficiency syndrome (AIDS) dementia complex (Price *et al.*, 1988).

The major known function of microglia is the phagocytosis of cellular debris, which may be important for tissue modeling in the developing CNS (Perry & Gordon, 1988). Also, microglia may be involved with inflammation and repair in the adult CNS due to their phagocytic ability, release of neutral proteinases and production of oxidative radicals. Microglia processes are incorporated in the layer of astrocytic foot processes of the perivascular glia limitans, and thus may contribute to the integrity of the blood brain

barrier (BBB) (Lassmann *et al.*, 1991). Moreover, microglia have been demonstrated to express major histocompatibility complex (MHC) antigens upon activation, act as antigen presenting cells, secrete a number of immunoregulatory cytokines, and respond to cytokine stimulation, suggesting an involvement with immune response within the CNS (Benveniste, 1992).

1.7.3 Astrocytes

Named for their starry shape, astrocytes have been known since the late 19th century. The astroctye is the most abundant cell type in the CNS, outnumbering neurons by about 10:1. They are, in reality, a lineage representing a large family of cells that share certain biochemical and morphological specialization, while diverging in certain functional capabilities.

Classically, there are two principal types of astrocytes, the protoplasmic and fibrous astrocytes, which are classified according to their morphological properties, antigenic phenotypes, kinetic development, appearance, and response to the growth factors. The protoplasmic astrocytes are characterized by thick, branched processes with spiny projections and are localized primarily within the gray matter. The fibrous astrocytes, in contrast, consist of relatively long, thin processes with few branches and are the predominant type in the white matter.

In the past, it was assumed that astrocytes serve as little more than passive physical support elements for neurons in the CNS. This simple view has given way as advances in astrocyte biology have made it possible to understand their functions better. It is now clear that astrocytes play important roles in brain development and in the

pathology of the nervous system. It has been argued that the brain will never be fully understood without an understanding of the many roles of astrocytes (Kimelberg & Norenberg, 1989).

Fundamentally, there are three biochemical characteristic features that define astrocytes in the mammalian CNS. The most important is the cytoplasm inclusion of 6- to 9-nm intermediate filaments whose major structural component is a 49-kD protein, the glial fibrillary acidic protein (GFAP) (Bignami *et al.*, 1972). Recently, other biochemical markers in astrocytes are also found, such as glutamine synthetase (GS), the calcium binding protein, S-100 (Isobe & Okuyama, 1978; Norenberg & Martinz-Hernandez, 1979; Moore, 1965; Ghandour *et al.*, 1981). The presence of these two enzymes suggest that astrocytes play important role in glutamate/glutamine metabolism and calcium homeostasis in the CNS.

1.7.3.1 Functions of astrocytes

Along with the great variety of astrocyte types, there is an equal diversity of specified functions that can be ascribed to particular astrocytes based upon their position relationships within the neuraxia and some of the general functions are:

a. Homeostasis

Astrocytes serve to maintain the composition of the fluid in the extracellular space of the brain. The homeostatic functions which are most directly pertinent to injury are regulation of extracellular K^+ concentration, participation in CO_2

metabolism, and clearance of neurotransmitter from the extracellular space. Astrocytes are involved in several of the mechanisms that modulate local blood supply in response to neuronal needs. Finally, astrocytes appear to provide substrates for energy metabolism to neurons (Benveniste, 1992).

b. Contribution to regulation of local blood flow

As the K^+ is derived from neuronal action potentials, it serves as an index of local neuronal activity as evidenced by observations that muscular blood vessels dilate when bathed in K^+ (Benveniste, 1992). It has been suggested that astrocytes serve as the conduit for the potassium would elicit a faster response to K^+ signaling by diffusion through the extracellular space (Benveniste, 1992).

The astrocytic participation in CO_2 metabolism results in the delivery of protons to the vicinity of endothelial cells, which would trigger the alterations of cerebral blood flow. In this interaction, proton concentration serves as a signal of neuronal aerobic metabolism, and presumably complements the potassium signal of neuronal action potentials (Benveniste, 1992).

c. Neurotransmitter transport system

One of the best-studied systems involves the metabolism of γ-aminobutyric acid (GABA), an inhibitory transmitter, which is metabolized by the enzyme GABA transaminase (Hansson *et al.*, 1984). Astrocytes have a high-affinity uptake system and metabolism of GABA, have a high level of GABA transaminase, and are thought

to be physically important for removing GABA from synaptic clefts (Schousboe *et al.*, 1977; Hertz *et al.*, 1978). Astrocytes are also involved in the metabolism of glutamate via the action of glutamine synthetase (Norenberg, 1979).

d. Mechanical support of neurons

The astrocyte provides a nonrigid supporting and insulating matrix for neurons. Because of their large numbers in the CNS, and the strength of the cytoplasmic projections (due to the presence of intermediate filaments within astrocytes), the astrocyte provides a supportive framework for nerve cells within the CNS (Benveniste, 1992).

e. Immunocompetent cells in the CNS

Studies in the past 10 years have demonstrated that astrocytes may be involved in immunological events occurring in the brain. The astrocyte, upon stimulation, can be induced to express MHC antigens and secrete the cytokines: IL-1, IL-6, TNF-α, and colony-stimulating factors; all are molecules that stimulate the growth and differentiation of macrophages and lymphoid cells. As such, the astrocyte has several important functional characteristics unique to traditional APCs, demonstrating the astrocytes' potential to act as immunocompetent cells in the CNS (Benveniste, 1992).

f. Guidance of migrating neurons during development

Migrating neurons use radial glial cells to aid in their placement (Rakic, 1971). Radial glia are astrocyte-related cells which appear during brain development, and then become transformed, presumably into GFAP-positive protoplasmic astrocytes (Schmechel & Rakic, 1979). *In vivo*, immature migrating neurons will cling closely to radial glia, and migrate along them to their final destination. It is thought that radial glia express adhesion molecules specific for developing neurons that allow them to arrive at their appropriate location in the brain (Choi & Lapham, 1976; Benveniste, 1992).

g. Induction of blood brain barrier (BBB)

The capillary endothelial cells that make up the BBB are joined by tight junctions; as a result, the BBB is virtually impermeable to soluble substances. The CNS capillaries are almost completely surrounded by astrocytic end-feet, thus, the astrocyte can contribute to the structural integrity of the BBB. Some recent evidence also suggests that the formation of tight junctions between the endothelial cells is induced by astrocytes contacting the endothelium (Benveniste, 1992).

h. Astrocytic reaction to injury

One of the most remarkable characteristics of astrocytes is their vigorous response to diverse neurologic insults, a feature that is well-conserved across a

variety of different species. The prominence of astroglial reactions in various neurologic disorders, such as head injury and infection diseases, the rapidity of the astroglial response and the evolutionary conservation of reactive astrocytosis indicate that reactive astrocytes fulfill important functions for the CNS (Eddleston & Mucke, 1993).

In response to brain injury, astrocytes extend numerous processes to form scar tissues a process called reactive gliosis, or astrogliosis. Reactive astrocyte formation is a nearly universal reaction to injury in the adult mammalian CNS, but it varies in intensity in different regions. A dense scar composed primarily of hypertrophic astrocytes forms rapidly at the site of a brain or spinal cord injury. Scar formation is thought to be important for recovering the tensile strength of the tissue and to shield intact parts of the CNS from secondary injury (Ridet *et al.*, 1997). The astrocytic scar is very compact, which has led to the suggestion that it may act as a physical barrier to axonal growth. The role of scar formation in axonal regeneration has, however, been difficult to establish and remains controversial (for reviewed see Frisén, 1997 and Ridet *et al.*, 1997; Pekny *et al.*, 1999).

The primary signals triggering the characteristic hypertrophy and increase of GFAP in reactive astrocytes have not been identified, but cytokines and trophic factors produced by microglia or invading monocytes and polymorphonuclear leukocytes can amplify the process (Perry & Gordon, 1988). Moreover, *in vitro* studies have identified a variety of neurotransmitter receptors, such as β-ARs and transporters (Shao & McCarthy, 1994), the high affinity uptake of glutamate, aspartate, GABA, and taurine, and nonsaturable uptake of norepinephrine (NE), dopamine and 5-hydroxytryptamine,

have also been characterized in primary astrocyte cultures (Hansson, 1989; Griffith Sutin, 1996). However, it is not clear whether these neurotransmitter receptors and/or uptake systems, with the exception that the β-adrenergic system, has been shown to play a significant role in brain injury (Griffith & Sutin, 1996; Hodges-Salova, *et al.*, 1996).

1.8 Brain injury, astrogliosis and scar formation

Following brain injury, there are often changes in morphology and shape in astrocytes and this process is known as reactive astrogliosis. This reaction is believed to be the regenerative outcome of CNS injury. Some of changes observed are hypertrophy of the cell bodies and cytoplasmic processes (Eng, 1987; Miller *et al.*, 1986; Norenbery, 1994) and an increase expression of GFAP (Bignami & Dahl, 1976; Schacher *et al.*, 1977), which culminating to form glial scar. Thus, astrogliosis can be readily measured by the increase in GFAP. However, the molecular signals or pathways that trigger the transformation of normal astrocytes to reactive astrocytes remain undefined. Recently, there is increasing evidence that growth factors (for example, colony stimulating factor), cytokines (for example, TNF-α), neurotransmitters (for example, noradrenaline) and neuropeptides may be involved (Hodges-Salova *et al.*, 1996). Among these substances, TNF-α and noradrenaline have attracted most attention. The possible role of TNF-α in this process has been discussed in **Section 1.4**.

One piece of the earlier evidence suggesting noradrenaline may be involved is the study with C6 glioma cells, which have many properties similar to cultured astrocytes. It was reported that the addition of N^6 -2'-dibutyryl cyclic adenosine-3', 5'-monophosphate (dbcAMP), an analogue of cyclic adenosine-3', 5'-monophosphate

(cAMP) resulted in an increase in GFAP synthesis and also the number of cells increased significantly (Messens & Slegers, 1992). cAMP is a well-known mediator of noradrenergic mechanism.

In addition, GFAP immunofluorescence in crushed nerves was reported to be dramatically elevated compared with intact optic nerves (Hodges-Salova *et al.*, 1996). More interestingly, β-agonist isoproterenol (ISO) also promoted changes in GFAP in the absence of injury, while propranolol, an β-antagonist, suppressed the injury-induced GFAP immunoreactivity (Hodges-Salova *et al.*, 1996). These demonstrated that nerve injury caused a significant increase in GFAP immunoreactivity and this mechanism is liked mediated through an adrenergic mechanism.

β-Adrenergic substances exert their actions by binding to β-ARs, and some of their properties are briefly described in **Section 1.9**.

1.9 β-Adrenergic receptors (β-ARs)

β-Adrenergic receptors (βARs) are expressed by astrocytes and three β-adrenergic receptors, β1-, β2- and β3-ARs, are now known to bind norepinephrine and epinephrine, to couple to the G_S stimulatory GTP binding protein, and stimulate adenylyl cyclase to produce cAMP. While these three receptors display quite similar overall structure and function (Strosberg, 1993), a number of intriguing differences have been described, most probably linked to specific amino acid sequence variations of the receptors (Strosbery, 1995).

1.9.1 The active functional unit: the receptor complex

The human β-AR genes are situated on the long arm of chromosome 5 and codes for an intronless gene product of approximately 1200 base pairs (Johnson, 1998).

The receptor is a member of the seven-transmembrane family of receptors. It is composed of 413 amino acid residues of approximately 46 kDa (Henderson *et al.*, 1990). β-ARs have been subdivided into at least three distinct pharmacological and molecular subtypes: β_1 ,β_2 and β_3 (Johnson, 1998).

The functional response produced by catecholamine depends on subsequent interactions between these components: (i) the catecholamines bind to the receptor and convert it to its active form; (ii) the activated receptor interacts closely with the nearby G protein, which in turn becomes active; and (iii) the activated G protein in turn activates the enzyme adenylate cyclase. The latter, then converts adenosine triphosphate (ATP) to cAMP, which acts as an intracellular second messenger (De Blasi, 1990).

1.9.2 General functions and distribution of β-ARs

Of the β-receptors, β_1-adrenergic receptors (β1-ARs) predominate in the heart and in the cerebral cortex, whereas β_2-adrenergic receptors (β2-ARs) predominate in the lung and cerebellum. However, in many cases, both β1- and β2-AR coexist in the same tissue, sometimes mediating the same physiological effect (De Blasi, 1990).

A third subtype of β-adrenergic receptor has also been identified. This receptor has pharmacological properties distinct from those of β1- or β2-AR. Agonists that are selective for β3-ARs exist and cause nonshivering thermogenesis in rodents (Strosberg,

1995). The role of this receptor in humans remains to be defined. The mRNA for β3-AR is selectively expressed in brown adipose tissue in rodents and in newborn humans. Message can also be detected in white adipose tissue, but the level of expression is very low (Strosberg, 1995). Because of its distinct localization in adipose tissues, it is likely that this receptor subtype is highly related to lipid metabolism.

The brain contains both β1- and β2-ARs, which cannot be differentiated in terms of their physiological functions. The density of β1-AR varies in different brain areas to a greater extent than does that of β2-AR. It has been suggested that this is due to the high density of β2-AR in glia or blood vessels (Strosberg, 1995).

Interestingly, interactions between β-ARs and cytokines have been reported in peripheral tissues. Hetier *et al* (1991) and Severn *et al* (1992) observed that β-adrenergic agonists could prevent the lipopolysaccharide (LPS) stimulation of TNF-α production by microglia and whole blood cells and THP-1 cells (a kidney cell line). These data suggest that β-AR activation might be able to suppress cytokine production in the kidney. Indeed, Nakamura *et al.* (1998) reported that isoproterenol depressed renal IL-6 and TNF productions under conditions when angiotensin II generation was inhibited. But, the mechanisms underlying the abnormal cytokine production in the spontaneously hypertensive rat are poorly understood. Moreover, Yoshimura *et al.* (1997) reported that β-agonists inhibit the production of TNF-α and IL-1β by LPS-stimulated human peripheral blood mononuclear cells and that the inhibitory effects depend upon the elevation of cAMP levels. However, interactions between β-ARs and cytokines are still unclear.

1.10 Functions of β-ARs in astrocytes

Though astrocytes have been found to express β1- and β2-AR, the functions of β-ARs in astrocytes are still controversial.

1.10.1 Regulation of astrogliosis by β-ARs

Accumulated evidence suggests that β-AR in astrocytes can regulate astrogliosis, and some supporting reports are briefly summarized below:

1.10.1.1. β-ARs are expressed in normal optic nerves and up-regulated after nerve crush

Astrocytes in culture, as well as in various regions of the mammalian CNS, have been shown to express β-ARs (Mantyh *et al.*, 1995; Salm & McCarthy, 1989; 1992; Sutin & Shao, 1992), and Mantyh *et al.* (1995) also demonstrated that β-ARs are co-localized with astrocytes in normal rat, rabbit, and human optic nerves (Mantyh *et al.*, 1995). Moreover, Hodges-Savola *et al.* (1996) showed that a marked increase in the concentration of β-ARs 14 days after rabbit optic nerve crush and that Mantyh *et al.* (1995) showed that, in rats as well as rabbits, a significant increase in the density of β-AR occurred within the first 30 days after nerve transection and sustained for 90 days. These studies clearly show that β-ARs are up-regulated, expecially in astrocytes, of injured nerves. However, the mechanism is not known.

1.10.1.2 Injury-induced alterations in endogenous catecholamines leads to enhanced β-AR activation

Under normal conditions, the BBB should effectively prevent passage of physiologically significant amounts of catecholamines from the circulation into the parachyma of the brain (Cryer, 1987). However, following head injury/trauma, human plasma catecholamine levels increased 4-5 fold (Hamill *et al.*, 1987) due to BBB disruption in the region of the injury (Cancilla *et al.*, 1993). Taken together, such alterations could allow substantially elevated levels of endogenous catecholamines access to β-ARs at or near the injury site. The resulting increase in the levels and availability of noradrenaline at the site of injury would subsequently initiate the sequence of events leading to astroglial hypertrophy, cellular proliferation, and scar formation (Hodges-Savola *et al.*, 1996).

1.10.1.3 β-AR blockade suppresses glial scar formation

Recently, Sutin and Griffith (1993) reported that continuous *in vivo* treatment of the β-antagonist propranolol led to a reduction in the GFAP-immunoreactivity intensity following ricin-induced motor neuron degeneration in the rat spinal cord. This suggests that reactive gliosis *in vivo* and also the astrocyte hypertrophy could be reduced by β-adrenergic antagonists. Moreover, Hodges-Savola *et al.* (1996) demonstrated that propranolol infusion significantly reduced the crush-induced increase in GFAP immunofluorescence and also attenuated the concomitant increase in cell numbers within

injured nerves. As a result, these investigators suggest that the β-adrenergic mechanism is involved in the regulation of astrogliosis after CNS injury.

1.10.1.4 β-AR agonists affect the proliferation of astrocytes in normal brain

Recently, Imura *et al.* (1999) reported that isoproterenol increased heat shock protein 27 (HSP27) and mimicked the expression of small heat shock proteins (sHSPs) in reactive astrocytes *in vivo*. This effect has been observed in both C6 glioma cells and primary astrocytes with or without ischemia. The formation of reactive astrocytes *in vivo* seems to depend on the overexpression and reorganization of cytoskeletal proteins, such as GFAP and actin (Imura *et al.*, 1999). Previous studies have revealed that sHSP can modulate not only actin microfilament dynamics (Lavoie *et al.*, 1993) but also GFAP assembly (Nicholl & Quinlan, 1994). β-AR activation has been shown to increase the synthesis of GFAP in astrocytes (Segovia *et al.*, 1994) and also to regulate GFAP assembly by the phosphorylation of their non-α-helical head domains (McCarthy *et al.*, 1985; Ralton *et al.*, 1994). These findings suggest that β-AR activation and an increase in HSP27 may play an important role in cytoskeletal reorganization, accompanied by the formation of gliosis after ischemic injury. These observations also help to explain the changes in astrocyte morphology following injury. Moreover, Hodges-Savola *et al.* (1996) demonstrated that β-AR activation, in the absence of injury, can promote astroglial hypertrophy and cell proliferation. They also found that there was a significant increase in both GFAP-immunoreactivity and cell number in non-injured nerves infused with isoproterenol. Interestingly, the magnitude of change in isoproterenol-infused nerves was similar to those detected in crushed nerves. These data suggest that β-ARs

directly influence astrocyte function and that injury-related alterations in the amount and/or availability of endogenous catecholamines lead to enhanced β-AR activation in astrocytes.

1.11 Manganese Superoxide Dismutase (MnSOD)

Evidence presented in the above sections suggests that TNF-α exerted a proliferative effect on astrocytes and C6 glioma cells, but TNF-α is cytotoxic to other cell types, such as tumor cell lines (Sugarman *et al.*, 1985). The differential cellular sensitivities to TNF-α are not clear at present. In this connection, it is interesting to note that Wong *et al.* (1989) reported that the generation of superoxide radicals (O_2^-) or other reactive oxygen species (ROSs) that are capable of damaging cellular components (Freeman & Crapo, 1982) and activating the release of certain enzymes, such as lysosomal enzymes (Smolen, 1984), might be involved in the TNF-α-initiated cytotoxic pathway (Jones, 1986).

O_2^- and hydrogen peroxide (H_2O_2) are producing by mitochondria during electron transport (Boveris, 1977; Freeman & Crapo, 1982; Forman & Boveris, 1982). Other intracellular sources of O_2^- and H_2O_2 are endoplasmic reticulum, peroxisomes, and nuclear and plasma membranes (Freeman & Crapo, 1982). Protection against ROS toxicity requires antioxidants, such as glutathione and sulfhydryl components, the H_2O_2-removing enzymes catalase and glutathione peroxidase, and the O_2^- scavenging enzyme superoxide dismutase (SOD) (Hassan, 1988; Touati, 1988; Wong *et al.*, 1989).

Mitochondria, a major subcellular source of ROS (Dugan *et al.*, 1995; Piantadosi & Zhang, 1996), may play pivotal roles in apoptosis (Kroemer *et al.*, 1997). MnSOD is

an inducible enzyme that plays a crucial role in the oxidative damages caused by superoxides and other ROSs in mitochondria under physiological, hypoxia-reperfusion, and hyperoxic conditions. The precise mechanism of the induction of MnSOD gene expression is not known (Pahan *et al.*, 1999), and much less is known of its relationship with elevated cytokines, e.g., TNF-α.

MnSOD is a superoxide dismutase associated specifically with mitochondria in which it captures and reduces free radicals, preventing oxidative damage to mitochondria and surrounding organelles. Free radical damage has long been held as a key element in promoting neuronal cell death in CNS trauma (Braughler & Hall, 1989; 1992; Hall & Braughler, 1989; 1993), whereas, overexpression of MnSOD has been shown to prevent neuronal cell death by suppression of peroxynitrite production and lipid peroxidation (Keller *et al.*, 1998). MnSOD deficiency may contribute to the increase of cytosolic release of cytochrome c and to DNA fragmentation that follows (Fujimura *et al.*, 1999). These support the protective role of MnSOD in cell injury.

MnSOD expression and increased resistance to cell injury and death have been established in several paradigms, including resistance of tumor cells to killing by TNF-α (Wong & Goeddel, 1988), and from resistance of cardiac myocytes treated with TNF-α to ischemic injury (Nelson *et al.*, 1995; Keller *et al.*, 1998). Despite such correlations, it is not known whether and how MnSOD exerts an anti-apoptotic function in these tissues/organs.

1.11.1 MnSOD is the target gene of NF-κB

TNF-α receptor activation results in activation of the transcription factor nuclear factor κB (NF-κB) which may serve an antiapoptotic role via the induction of target genes MnSOD (Tan *et al.*, 1994). There is an emerging body of evidence implicates a consequential role for NF-κB based on its activation in various brain injury paradigms (Devary *et al.*, 1993; Meyer *et al.*, 1994; Prasad *et al.*, 1994; McIntosh & Raghupathi, 1995; Yang *et al.*, 1995; O'Neill & Kaltschmidt, 1997; Mattson *et al.*, 1997). One of the possible target genes of NF-κB activation via TNF-α is that of the antioxidant MnSOD, which is upregulated in response to TNF-α in a variety of cell types (Das *et al.*, 1995; Wong, 1995; Wong *et al.*, 1996; Hachiya *et al.*, 1997; Isoherranen *et al.*, 1997; Jones *et al.*, 1997).

1.11.2 Induction of MnSOD by proinflammatory cytokines in rat primary astrocytes

Bacterial lipopolysaccharide (LPS) and proinflammatory cytokines are known to induce the degradation of SMase to ceramide in rat primary astrocytes (Pahan *et al.*, 1998; Singh *et al.*, 1998). It has also been reported that LPS and different cytokines induce MnSOD in different peripheral cell types (Wong *et al.*, 1989). Recently, Pahan *et al.* (1999) demonstrated that LPS and some cytokines also induced the activity of MnSOD by more than twofold in primary astrocytes. However, the mechanism remains to be established.

Moreover, Wong *et al.* (1989) have also shown that treatment with TNF-α induces the expression of mRNA for MnSOD but not for other antioxidant enzymes,

such as CuZnSOD, catalase and glutathione peroxidase (Wong & Goeddel, 1988). This suggests the induction is selective. Furthermore, treatment of kidney cells with IL-1 was found to induce the expression of MnSOD (Wong & Goeddel, 1988; Masuda *et al.*, 1988), and to inhibit the cytotoxicity of TNF-α (Holtmann & Wallach, 1987). Thus, MnSOD is likely a candidate for protecting cells from the cytotoxicity of TNF-α.

1.11.3 SMase and ceramides induce MnSOD in various cell types

Ceramide has been shown to be an important inducer of apoptosis in different cell types (Obeid *et al.*, 1993; Hannun, 1996). However, Pahan *et al.* (1999) has reported that activation of SMase or addition of ceramide in astrocytes increased the activity of MnSOD, and the mRNA level of MnSOD was also induced.

Therefore, the induction of MnSOD by ceramide suggests that apart from transducing an apoptotic signal in the cell, ceramide also induces a protective signal for the enhanced elimination of cytotoxic superoxide radicals via induced MnSOD. Probably, the degree of ceramide-induced apoptosis is dependent on the level of ceramide-induced MnSOD activity: that is the higher the ceramide-induced expression of MnSOD activity, the lower the ceramide-induced apoptosis and vice versa (Pahan *et al.*, 1999). Consistent with this idea is the finding of Keller *et al.* (1998) who reported that mitochondrial MnSOD prevented neural apoptosis and reduced ischemic brain injury. As TNF-α induces C6 glioma cell proliferation (Liu, 1996), it is important to determine whether MnSOD is a candidate responsible for the anti-apoptotic and/or the proliferative effect(s) of TNF-α and the mechanism(s) involved.

1.12 Why do we use C6 glioma cells?

The term glioma describes the group of glial neoplasma including astrocytoma, glioblastoma, ependynoma, oligodendroglioma and mixed gliomas, such as oligoastrocytoma. Glioma cells are particularly easy to grow in tissue culture (Westermark *et al.*, 1973). Although they may not be perfect representatives in all aspects of glioma or glial cells *in vivo*, the relative ease with such cell lines can be established has made them widely used as a model system for studies on the biology of glial cells. A number of rat brain tumors induced by N-nitrosomethylurea and consisting of more or less differentiated astrocyte-like cells are found to contain S-100 protein, a characteristic protein of glial cells. It was shown that injection of newborn rats with these cultured tumor cells resulted in a high efficiency of tumor formation (Benda *et al.*, 1968). Five morphologically distinct clonal cell strains were established from these tumors, only one contains appreciable amounts of S-100, and this is the C6 glioma cell line (Benda *et al.*, 1968).

C6 Glioma cells have provided a useful model to study glial cell properties, glial factors and sensitivity of glial cells to various substances and conditions (Kempski *et al.*, 1992; Vernadakis *et al.*, 1992). As a kind of transformed cell line, the growth characteristics of C6 cells are immortal, anchorage independent, loss of contact inhibition, high plating efficiency and shorter population doubling time. In addition to its homogeneous genetic properties, C6 cells are tumorigenic and angiogenic (Freshney, 1987). C6 Cells express several glial specific markers, such as S-100, GFAP and GS, the markers for astrocytes, as well as CNP, an enzyme marker for oligodendrocytes (Kempski *et al.*, 1992; Vernadakis *et al.*, 1992). Moreover, the expression or activity of

these markers in C6 cells could be induced. For instance, CNP is induced by neuron-derived factors, epidermal growth factor and fibroblast growth factor, whereas GFAP and GS are induced by insulin, cyclic AMP, platelet-activating factor, muscle-derived factors, chronic β-receptor activation and interleukin-4 (IL-4) (Brodie & Goldreich, 1994; Brodie & Vernadakis, 1991; Parker *et al.*, 1980). Interestingly, cytokines are known to be involved in astrocytic and oligodendrocytic properties expression in C6 cells of early passages (Brodie & Goldreich, 1994).

C6 Glioma cells have been found to respond to several cytokines, such as TNF-α, IL-6, interferon-γ (IFN-γ) and IL-4. The growth of C6 glioma cells is stimulated by the TNF-α, IL-6 and IFN-γ (Munoz-Fernandez *et al.*, 1991; Munoz-Fernandez & Fresno, 1993). In addition, IL-4 exerts a biphasic effect on C6 cell proliferation, increasing cell proliferation at concentrations ranging from 10-50 ng/ml, while exhibits inhibitory effect at higher concentrations (Brodie & Goldreich, 1994). The inhibition of cell proliferation is associated with differentiation of the cells to express astrocytic phenotypes as evidenced by morphology, increased GFAP immunoreactivity and elevated GS expression (Brodie & Goldreich, 1994). IL-4 also induces the secretion of nerve growth factor in C6 glioma cells (Brodie & Goldreich, 1994). These results suggest that the C6 cell line represents a good cell model for the study of the proliferation and differentiation of glial cells *in vitro*.

1.13 Aims and Scopes of the project

The CNS has traditionally been regarded as an immunologically privileged site. However, recent findings showed that certain cells of the CNS, particularly astrocytes,

are modulated by various cytokines, including TNF-α (Benveniste, 1992). Injury to the CNS results in the formation of the glial scar, an astrocytic process closely associated with astrocyte proliferation, that obstructs regrowing axons. In fact, a number of *in vitro* experiments have shown that astrocyte proliferation can inhibit axon growth (Asher *et al.*, 2000).

Recently, it was found that increased levels of TNF-α were observed in plasma and CSF in patients after brain injury, and acute increases in TNF-α mRNA and protein expression have also been observed in injured rat brain (Fan *et al.*, 1996; Scherbel *et al.*, 1999). Furthermore, anti-TNF-α-substances, for example, HU-211 or TNF-α binding protein, improve the neurological outcome in victims of brain injury (Shohami *et la.*, 1997). So, TNF-α, produced largely by astrocytes of the injured brain, may play an important role in the elevation of TNF-α and astrocytes proliferation, and possibly scar formation observed in brain injury. Consistent with this thought is the report that astrocytes undergo dramatic metabolic and morphologic changes, or commonly called "reactive astrogliosis" in response to trauma or other pathological insults in the CNS (Hodges-Savola *et al.*, 1996). In addition, β-ARs have been found to regulate astrocyte proliferation and/or astrogliosis (Hodges-Savola *et al.*, 1996; Sutin & Griffith, 1993). So, TNF-α could mediate certain pathological events after brain injury via β-AR mechanism. But, the relationship between elevated TNF-α and β-adrenergic mechanism following brain injury is still undefined. As a result, it is important to investigate relationship between these events with C6 glioma cells so as to elucidate the signal transduction pathway(s) mediating the proliferative effects of TNF-α and β-adrenergic mechansim in C6 glioma cells as well as in astrocytes. Astrocytes are capable of synthesize and secrete cytokines and they are the most abundant cell type in the CNS.

The long-term goal is to find better therapeutic means to improve the outcome of brain injury.

As diverse intracellular signaling pathways have been proposed to regulate the expression of cytokines in peripheral cells and that several second messenger systems have been reported to mediate cytokine-induced responses, therefore, in the first set of experiments, the intracellular signaling pathways mediating the expression of TNF-Rs, as well as that β-ARs in C6 glioma cells were examined. As PKC has been found to be an important signal transduction pathway in brain injury (Zablocka *et al.*, 1998) and it is also a common second messenger system in many cell types, therefore, this pathway was investigated. Moreover, this enzyme has been found in C6 glioma cells (Tsang *et al.*, 1997). A PKC activator, 12-myristate 13-acetate (PMA) and a specific PKC inhibitor, 3-{1-[3 (amidinothio) propyl]-3-indolyl}-4-(1-methyl-3-indolyl)-1H-pyrrole-2, 5-dione methanesulfonate (Ro-31) (Tsang *et al.*, 1997) were used to study the role of PKC on TNF-Rs and β-ARs expression. Moreover, as PKA is also a common second messenger system in C6 cells (Messens & Slegers, 1992), its activator, dbcAMP was used to investigate whether PKA is involved between TNF-α and induction of TNF-Rs and β-ARs expression.

In the second set of experiments, the relationship between β-ARs and TNF-Rs was examined. In this study, different selective β-adrenergic agonists and antagonists were examined for their effects on TNF-α and TNF-R expression.

MnSOD induction has been found to be important for protection against oxidative stress, and that MnSOD can block or delay apoptosis (Cai & Jones, 1998). Since earlier work in our laboratory showed that TNF-α can stimulate C6 proliferation (Liu, 1996), thus, in the third set of experiments, we investigated the effect of TNF-α on

the MnSOD expression so as to elucidate the relationship between TNF-α, β-adrenergic mechanism and MnSOD expression. As previous findings in our laboratory suggested that TNF-α action was mediated through β-AR activation, we examined the effects of some β-adrenergic agonists and antagonists on MnSOD expression. Moreover, as MnSOD was found to be one of the target genes of NF-κB (Das *et al.*, 1995; Mattson et al., 1995; Wong, 1995) and that we found TNF-α induced NF-κB expression (Lung, 1999), so the effects of some β-adrenergic agonists and antagonists were also included.

The major aim of this study is to elucidate the signaling pathway mediating astrocyte proliferation, an important step that leads to astrogliosis. Last but not least, it is hoped that results obtained would provide valuable information on the regulatory mechanisms leading to astrogliosis which should lead to a more effective treatment for brain injury.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Line

Rat C6 glioma cells were obtained from the American Type Culture Collection (U.S.A.). They were originally cloned from a rat glial tumor induced by N-nitrosomethylurea after a series of alternate culture and animal passages (Benda *et al.*, 1968).

2.1.2 Cell Culture Reagents

2.1.2.1 Complete Dulbecco's modified Eagle medium (CDMEM)

Dulbecco's modified Eagle medium (DMEM) with glucose and L-glutamine was purchased from Gibco BRL (U.S.A.). The powdered DMEM (for 1 litre solution) and 3.7 g sodium bicarbonate were dissolved in 1 litre of double-distilled water. The medium was adjusted to pH 7.2 and filtered (filter: 0.2 μM, Micro Filtration Systems, Dublin, U.S.A.) immediately under suction. Then, heat-inactivated fetal bovine serum (HI-FBS; purchased from Gibco BRL, U.S.A.), and antibiotics (penicillin, 10,000

U/mL; streptomycin, 10,000 μg/mL; fungizone, 25 μg/mL; Gibco BRL, U.S.A.) were added to make the complete DMEM (CDMEM). The final CDMEM contained 10% (v/v) HI-FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone. This medium was stored at 4°C until use.

2.1.2.2 Rosewell Park Memorial Institute (RPMI) medium

Rosewell Park Memorial Institute (RPMI) medium was prepared almost the same as DMEM, except that 0.2 g sodium bicarbonate was added. The final complete RPMI (CRPMI) medium contained 10% (v/v) HI-FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone. Because RPMI medium contained N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) as a buffering system, its pH is more stable than DMEM, hence, the CDMEM was used in the maintenance of cell culture, while the CRPMI medium was used for proliferation assays. This medium was stored at 4°C until use.

2.1.2.3 Phosphate buffered saline (PBS)

Phosphate buffered saline (PBS) was prepared by dissolving 8.18 g sodium

chloride, 0.2 g potassium chloride, 0.2 g potassium dihydrogen phosphate and 1.44 g sodium hydrogen phosphate in 1 litre of nano-pure water, and the pH of PBS was adjusted to 7.4 then autoclaved.

2.1.3 Recombinant cytokine

Recombinant mouse tumor necrosis factor-α (TNF-α, specific activity: 6×10^7 U/mL) was purchased from Boehringer Mannheim Biochemica (Germany). This cytokine had been shown to be biological active in C6 glioma cells (Huang *et al.*, 1998). It was stored in aliquots at -20°C until use.

2.1.4 Chemicals for signal transduction study

2.1.4.1 Modulators of protein kinase C (PKC)

Protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA; Sigma, U.S.A.) was dissolved in complete culture medium at a final concentration of 4 μM and stored at –20°C until use. PKC inhibitor, 3-{1-[3-(amidinothio)propyl]-3-indolyl}-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione methanesulfonate (Ro-31;

Calbiochem, U.S.A.) was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 100 μM. The dissolved PKC inhibitor was also stored at –20°C until use. Both modulators had been shown to be very selective in C6 glioma cells (Tsang *et al.*, 1997).

2.1.4.2 Modulator of protein kinase A (PKA)

Protein kinase A (PKA) activator, N^6-2'-dibutyryl cyclic adenosine-3',5'-monophosphate (dbcAMP; Sigma, U.S.A.), was dissolved in complete culture medium at a final concentration of 100 mM and stored at –20°C until use.

2.1.4.3 β-Adrenergic agonist and antagonist

Isoproterenol, an β-adrenergic receptor (β-AR) agonist, and propranolol, an β-AR antagonist, were purchased from Sigma, U.S.A. They were dissolved in double-distilled water at final concentrations of 250 mM and 10 mM respectively, and stored at –20°C until use.

i) β1-Adrenergic agonist and antagonist

Dobutamine, an β1-adrenergic receptor (β1-AR) agonist (Deighton *et al.*, 1992), and atenolol, an β1-AR antagonist (Koganei *et al.*, 1995), were purchased from Sigma, U.S.A. They were dissolved in double-distilled water at final concentrations of 10 mM and 50 mM respectively, and stored at -20 °C until use.

ii) β2-Adrenergic agonist and antagonist

Procaterol, an β2-adrenergic receptor (β2-AR) agonist (Koganei *et al.*, 1995), and ICI 118,551, an β2-AR antagonist (Deighton *et al.*, 1992), were purchased from Sigma, U.S.A. They were dissolved in double-distilled water and at a final concentration of 10 mM, and stored at -20 °C until use.

2.1.5 Antibodies

2.1.5.1 Anti-TNF-receptor type 1 (TNF-R1) antibody

Antibody against TNF-receptor type 1 (TNF-R1; 200 μg/mL), an affinity-

purified goat polyclonal antibody raised against a peptide corresponding to amino acids 26 to 45 mapping at the amino terminus of the TNF-R1 precursor of mouse origin, was purchased from Santa Cruz Biotechnology, Inc., U.S.A. According to the manufacturer, this antibody only reacts with TNF-R1 of mouse and rat origin by Western blotting and immunohistochemistry and is non-reactive with TNF-R2. This antibody was stored at 4°C until use.

2.1.5.2 Anti-TNF-receptor type 2 (TNF-R2) antibody

Antibody against TNF-receptor type 2 (TNF-R2; 200 μg/mL), an affinity-purified goat polyclonal antibody raised against a peptide corresponding to amino acids 442 to 461 mapping at the carboxy terminus of the TNF-R2 precursor of mouse origin, was obtained from Santa Cruz Biotechnology, Inc., U.S.A. According to the manufacturer, this antibody only reacts with TNF-R2 of mouse and rat origin by Western blotting and immunohistochemistry and is non-reactive with TNF-R1. This antibody was stored at 4°C until use.

2.1.5.3 Anti-β1-adrenergic receptor (β1-AR) antibody

Antibody against β1-adrenergic receptor (β1-AR; 100 μg/mL), an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 446 to 464 mapping at the carboxy terminus of the β1-AR precursor of mouse origin, was obtained from Santa Cruz Biotechnology, Inc., U.S.A. According to the manufacturer, this antibody only reacts with β1-AR of mouse and rat origin by Western blotting and immunohistochemistry and is non-reactive with β2- or β3-AR. This antibody was stored at 4°C until use.

2.1.5.4 Anti-β2-adrenergic receptor (β2-AR) antibody

Antibody against β2-adrenergic receptor (β2-AR; 100 μg/mL), an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 399 to 418 mapping at the carboxy terminus of the β2-AR precursor of mouse origin, was obtained from Santa Cruz Biotechnology, Inc., U.S.A. According to the manufacturer, this antibody only reacts with β2-AR of mouse and rat origin by Western blotting and immunohistochemistry and is non-reactive with β1- and β3-AR. This antibody was stored at 4°C until use.

2.1.5.5 Antibody conjugates

Goat anti-rabbit biotin conjugate and anti-biotin alkaline phosphatase conjugate were supplied by Tropix, Inc. (Massachusetts, U.S.A.). Monoclonal mouse anti-goat biotin conjugate (1 μg/mL) was purchased from Sigma, U.S.A. All antibody conjugates were stored at 4°C until use.

2.1.6 Reagents for RNA isolation

TRIzol reagent (Gibco, U.S.A.) and isopropanol (Promega, U.S.A.) were stored at 4°C, while chloroform (AnalaR, England) and ethanol (AnalaR, England) were stored at room temperature.

2.1.7 Reagents for reverse transcription-polymerase chain reaction (RT-PCR)

Deoxynucleotide mix (dNTP, 10 mM each dNTP), AMV reverse transcriptase (25 U/μL), RNAse inhibitor (50 U/μL), random primer $p(dN)_6$ (2 μg/mL), *Taq* DNA polymerase (produced in *E. Coli,* 5 U/μL) were purchased from Boehringer Mannheim, Germany. All restriction enzymes used in this study were purchased from

New England Biolabs, U.S.A. The following chemicals: Tris-ethylenediaminetetraacetic acid disodium salt (TE) buffer used to reconstitute and dilute PCR primers, the TE buffer contained 10 mM Tris (pH 8.0) and 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA) were purchased from Sigma, U.S.A.

The sets of specific primers for PCR amplification of the corresponding specific cDNAs were purchased from Gibco (Hong Kong) and summarized in Table 1. The primers were designed on the basis of previous published data TNF-R1 (Himmler *et al.*, 1990), TNF-R2 (Bader *et al.*, 1996), β-actin (Nudel *et al.*, 1983), β1-AR (Elalouf *et al.*, 1993), β2-AR (Gocayne *et al.*, 1987), nuclear factor-κB (NF-κB/p50) (Tavares *et al.*, 1998), manganese superoxide dismutase (MnSOD) (Sugino, *et al.*, 1998), copper-zinc superoxide dismutase (Cu-ZnSOD) (Sugino *et al.* 1998), TNF-α (Kwon *et al.*, 1993), and these oligonucleotides were derived from the sequences of the corresponding rat genes and cDNA, with nucleotide positions as indicated in Table 1. In addition, the primer sequences were chosen from separate exons of the genes so that the RT-PCR product could readily be distinguished from any genomic DNA-induced PCR products. Each primer was diluted to 2.5 μM and stored at –20°C until use.

2.1.8 Reagents for electrophoresis

Ten x DNA loading buffer contained 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 60 mM EDTA (pH 8.5). Ten x Tris-boric acid-EDTA (TBE) buffer was prepared by dissolving 108 g Tris base, 55 g boric acid and 7.44 g EDTA in 1 L double-distilled water, and the pH was adjusted to 8.3. All of the above chemicals were obtained from Sigma, U.S.A. DNA ladder purchased from Bio-Rad was used as markers.

2.1.9 Reagents and buffers for Western blot

I- Block™ (highly purified casein), CSPD® Ready-to-Use substrate solution (0.25 mM), Nitro-Block™ (20 x chemiluminescent enhancer), and protein molecular weight markers were supplied by Tropix, Inc. (Massachusetts, U.S.A.). Two x Loading buffer contained 0.5 M Tris, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol and 0.03% (w/v) bromophenol blue. One x Running buffer was made up of 0.025 M Tris, 0.19 M glycine and 0.1% (w/v) SDS. Transfer buffer contained 39 mM glycine, 48 mM Tris, 0.037% (w/v) SDS and 20% (v/v) methanol.

Table 1 Primers used in RT-PCR and the predicted sizes of the PCR products.

cDNA Amplified	Primer sequences (5'-3')	Nucleotide Position	Amplified PCR fragment size (bp)
TNF-R1	Sense strand: ACCAAGTGCCACAAAGGAACC Antisence strand: TACACACGGTGTTCTGTTTCTCC	415-435 714-736	 322
TNF-R2	Sense strand: ATGAGAAATCCCAGGATGCAG Antisence strand: ACAGACGTTCACGATGCAGGTG	2-22 232-253	 252
β-Actin	Sense strand: TGAGACCTTCAACACCCCAG Antisense strand: TTCATGAGGTAGTCTGTCAGGTCC	2166-2185 2343-2366	 201
β1-AR	Sense strand: TCGTGTGCACAGTGTGGGCC Antisense strand: AAGCGGCGCTCGCAGCTGTCA	533-552 774-794	 262
β2-AR	Sense strand: GCCAGCATCGAGACCCTG Antisense strand: TTTGTGCTCTTTCAAGCAGAAC	355-372 789-810	 456
NF-κB/p50	Sense strand: GAGATTCTGAATCCCCCTGA Antisense strand: TTCCAGCCGCTATGTGTAGA	4-23 807-826	 823
Cu-ZnSOD	Sense strand GCCGTGTGCGTGCTGAA Antisense strand: TTTCCACCTTTGCCCAAGTCA	9-25 371-391	 383
MnSOD	Sense strand: ATTAACGCGCAGATCATGCAG Antisense strand: TTTCAGATAGTCAGGTCTGACGTT	135-155 594-617	 483
TNF-α	Sense strand: TCCCAACAAGGAGGAGAAATT Antisense strand: TCATACCAGGGCTTGAGCTCAG	171-192 560-581	 411

PBS for preparation of blocking buffer contained 0.058 M sodium hydrogen phosphate, 0.017 M sodium dihydrogen phosphate and 0.068 M sodium chloride, and blocking buffer was prepared by dissolving 0.2% (w/v) I-Block™ (Tropix, Massachusetts, U.S.A.) and 0.1% (v/v) Tween®20 (Sigma, U.S.A.) in PBS. Wash buffer was PBS plus 0.1% (v/v) Tween®20, and assay buffer contained 20 mM Tris (pH 9.8) and 1mM magnesium chloride.

2.1.10 Other chemicals and reagents

All other chemicals were purchased from Sigma, U.S.A.

2.2 Maintenance of rat C6 glioma cell line

Rat C6 glioma cells were cultured, under aseptic conditions, in CDMEM at 37°C in a humidified incubator (Mode 2400, Shel-Lab., Inc.) under an atmosphere of 5% CO_2/95% air. The cells were sub-cultured after 2 to 3 days in culture, at which time they had reached confluence. All subculture procedures were performed under aseptic conditions in a culture hood (Biogard hood, Baker Company, Inc.) as follows: the medium was discarded, and cells were washed with sterile PBS. The cells were

treated with 1 mL of 0.25% (w/v) trypsin (Gibco BRL, U.S.A.) at 37°C for 5 minutes and then dispersed by shaking. After washed once with 10 mL CDMEM, the cells were adjusted to a cell density of 10^5 cells/mL and further cultured in a 75 cm^2 culture flask (Corning Laboratory Sciences Company, U.S.A.) containing 20 mL CDMEM.

For long-term storage, 1 mL of cells (10^7 cells) suspended in FBS containing 5% (v/v) dimethyl sulfoxide (DMSO, Sigma, U.S.A.) was stored in liquid nitrogen in plastic ampoules (Nunc., Denmark). When required, aliquots were thawed in CDMEM at 37°C, subcultured, and cells of passages 18 to 28 were used in this study.

2.3 RNA isolation

C6 Cells were seeded on 60 mm culture dishes and cultured with or without TNF-α and/or drugs as mentioned in **Section 2.2.2.** All RNA extraction procedures were done at 4°C. Cells were washed twice with 3 mL ice-cold PBS and 1 mL of TRIzol reagent was added to each dish. After 5 minutes, cells were scrapped off using a cell scrapper and transferred to an Eppendorf tube, then pipetted up and down several times to ensure cell breakage and RNA release. Afterwards, 0.2 mL of chloroform was added to each tube and followed by vortexing for 15 seconds. The tubes were allowed to stand at room temperature for 3 minutes, and centrifuged at

11,900xg for 15 minutes at 4°C. The supernatant was removed and 0.5 mL isopropanol was added, the solution was then vortexed thoroughly. The tubes were put into a freezer (-20°C) overnight to allow the RNA to precipitate. Then, the tubes were centrifuged at 11,900xg for 15 minutes at 4°C, and the pellet in each tube was washed with 1 mL 75% ethanol and centrifuged at 7,500xg at 4°C for 5 minutes. The RNA pellet was resuspended in 30 μL nuclease-free water and kept at -70°C until use.

2.3.1 Measurement of RNA yield

An aliquot of 4 μL RNA was mixed with 996 μL H_2O, and the absorbance at wavelength 260 nm (A_{260}) was measured to determine the concentration of RNA. The purity was determined by calculating the ratio of the absorbance at wavelength 260 nm to that at wavelength 280 nm (A_{260}/A_{280}). The yield and purity of RNA were calculated by the following equation:

$$\text{Yield} = \frac{A_{260} \times 40\ \mu g/\mu l}{(4/1000) \times 10^3}$$

$$\text{Purity} = A_{260}/A_{280}$$

All RNA samples were adjusted to 0.5 μg/μL, and this RNA concentration

was used for all reverse transcription (RT) reactions.

2.4 Reverse transcription-polymerase chain reaction (RT-PCR)

One μg of total RNA was reverse-transcribed in a 20 μL reaction mixture containing 20 U of AMV reverse transcriptase (Boehringer Mannheim, Germany), 5 mM $MgCl_2$, 1 mM of each dNTP (Boehringer Mannheim, Germany), 40 U of RNAse inhibitor (Boehringer Mannheim, Germany), 3.2 μg random primer $p(dN)_6$ (Boehringer Mannheim, Germany), 10 mM Tris/HCl, pH 8.3 and 50 mM KCl. The mixture was incubated at 25°C for 10 minutes, then 42°C for 60 minutes, heated to 99°C for 5 minutes and cooled to 4°C for 5 minutes. The RT samples were amplified immediately or stored at –20°C.

PCR was performed in a total volume of 25 μL consisting of 0.625 to 0.5 μL of RT sample (equivalent to 31.25-125 ng of total RNA), 0.625 U of *Taq* DNA polymerase (Boehringer Mannheim, Germany), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Boehringer Mannheim, Germany), 0.2 μM of both sense and antisense oligonucleotides (Gibco, Hong Kong), 10 mM Tris/HCl, pH 8.3 and 50 mM KCl in a PCR machine (GeneAmp PCR system 9700, Perkin Elmer, U.S.A.). The PCR conditions were: 94°C for 5 minutes, then 25 to 35 cycles of 94 °C for 0.5 minutes, 59

to 65 °C for 1 minute to 1 minute 15 seconds, 72 °C for 1 minute, followed by a final extension of 5 minutes at 72 °C. The sequences of PCR primers (Gibco, Hong Kong) were described in **Section 2.1.7** as summarized in Table 1.

A 5 μL-aliquot of PCR products was loaded with 6 x loading buffer and separated on a 2% (w/v) agarose gel. Each gel was run in 0.5 x TBE buffer at 125V for one hour, then stained with 1 μg/mL ethidium bromide solution and photographed under UV fluorescence, and the band density of the PCR product was quantified by densitometry using the program ImageQuant (Microsoft) by Molecular Dynamics. Density of the band from the target gene being analyzed was first normalized by the corresponding band from β-actin gene, then the normalized value of the treated sample was divided by the normalized value of the control in the same set of treatment to obtain a relative density value. The higher densitometry value suggested higher gene expression.

To ensure that all the PCR products were from the amplification of corresponding transcripts for the genes of interest, the PCR bands of the predicted sizes were further checked by restriction enzyme (RE) digestion to see if the PCR products contained the predicted restriction sites as described by Huang *et al.* (1998). For RE digestion, 20 μL of PCR products were digested with RE at 37°C for 4 hours and separated on a 2% (w/v) agarose gel. The results of RE digestion of MnSOD and

Cu-ZnSOD were shown in the **Appendix A**, while the other's had been reported previously (Huang *et al.*, 1996; Lung, 1999)..

2.5 Western blot analysis

To determine the receptor protein expression following cytokine and/or drug treatment, C6 cells were seeded at 6 x 10^5cells/100 mm-diameter dish in 10 mL of CDMEM. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air. On the next day, the culture medium was replaced with fresh medium. Stock solutions of individual cytokines and drugs were prepared in CDMEM and added to the culture medium to obtain the desired final concentrations. The time for the addition of drugs and/or cytokines was varied in such a way that all plates were harvested at the same time.

At the time of harvest, the culture medium was removed from the plate and cells were washed three times with 5 mL of PBS. One mL of Buffer A was added and cells were scrapped with a plastic scraper. Cells from 3 to 4 plates were combined into a 15 mL polypropylene centrifuge tube. After centrifugation at 1,000xg for 10 minutes, cell pellets were resuspended in 2 mL Buffer A and stored at –70°C until use.

Frozen cells were thawed at room temperature. Rupture of cells was achieved with sonication at 5 microns twice for 20 seconds (Soniprep 150, MSE SANYO). Membrane and lysate fractions were separated by centrifugation at 46,000xg for 30 minutes in a Beckman J2-MC centrifuge. The pellets containing membrane-chored receptors were resuspended in Buffer A and the protein contents were determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The membrane preparations were stored at –80°C until use.

Fifteen-twenty μg membrane preparations were loaded with 2 x loading buffer on a 10 to 15% polyacrylamide gel. The gel was run in 1 x running buffer at 100V for 1 1/2 hours. After electrophoresis, the gel, nitrocellulose membrane (Schleicher & Schuell) and Whatman filter paper were soaked with transfer buffer. The proteins on the gel were then electroblotted onto the nitrocellulose membrane by a semi-dry blotting unit (Bio-Rad). The blot was set up as follows: three pieces of Whatman filter paper were stacked on the platinum platform of a semi-dry blotting unit, the nitrocellulose membrane was placed on top of the filter paper stack, and the gel was placed on top of the membrane. Lastly, another three pieces of filter paper were stacked on the top. The transfer was run at a constant current of 30 mA per blot for two hours.

Following protein transfer, the blot was washed with PBS, and incubated in blocking buffer for one hour. The primary antibody specific to the protein of interest was diluted 1:1000 to 1:5000 in blocking buffer, and the blot was incubated with the diluted primary antibody overnight. The nitrocellulose membrane was washed twice for 5 minutes in a wash buffer. The biotinated secondary antibody was diluted 1:10,000 in blocking buffer, and the blot was incubated with the diluted secondary antibody for one hour. The nitrocellulose membrane was washed twice each for 5 minutes in a wash buffer. Anti-biotin alkaline phosphatase conjugate was diluted 1:20,000 in blocking buffer, and the blot was incubated with the diluted conjugate solution for 40 minutes. The nitrocellulose membrane was washed 3 times each for 5 minutes in a wash buffer, followed by washing twice each for 2 minutes with an assay buffer. Two millilitres CSPD® Ready-to-Use substrate solution (Tropix) containing 1:20 Nitro-Block™ (Tropix) was pipetted on the membrane, and the blot was incubated with the CSPD® solution for 5 minutes for the luminescent signal to develop. The nitrocellulose membrane was then exposed to a Kodak X-ray (XAR-5) film and the relative intensity of each band on the X-ray film was analyzed by ImageQuant (Microsoft) software on a densitometry system. The relative intensities is significanly changed when having ≥50% change comparing with control.

3. RESULTS

3.1 Effect of TNF-α on the expression of TNF-receptors (TNF-Rs) in C6 glioma cells

Some reports indicated that TNF-α produced by glial cells could play an important role during nerve tissue regeneration by stimulating the proliferation of glial cells (Selmaj *et al.*, 1990) as well as acting as an autocrine growth factor in inducing differentiation of neurons (For example, see Munoz-Fernandez *et al.*, 1991). However, very little is known about the TNF-α postreceptor signal transduction mechanism that leads to cell growth. Recent studies in our laboratory (Huang *et al.*, 1998) showed that TNF-α selectively induced the expression of TNF-R2 and that using selective TNF-R antibodies, TNF-R2 was found to be responsible for the proliferative effect in C6 cells (To, 1999), therefore the effect of TNF-α on TNF-R expression in C6 cells was studied. Moreover, attempts were made to elucidate the signal transduction pathways mediating TNF-α and its receptors expression.

To determine the effect of TNF-α on TNFR gene expression in C6 cells, the mRNA levels of both receptor subtypes were measured by RT-PCR as described in **Section 2.4**, and their protein levels determined by Western blot analysis as described in **Section 2.5**. RT-PCR was used to monitor TNF-R gene expression, as this method

is more sensitive than Northern and other techniques (Huang *et al.*, 1998). Western blot was applied to see if the proteins were expressed similar to their respective mRNA.

3.1.1 Effect of TNF-α on TNF-R1 and -R2 mRNA expression in C6 cells

A time-course experiment on the effect of TNF-α on both TNF-R1 and -R2 expression was performed. Fig. 4 showed the mRNA levels of TNF-R1- and -R2 in C6 cells after exposure to TNF-α (500 U/mL) for various time intervals. This concentration of TNF-α had been need to ensure there is a response at early time, 5 minutes. The β-actin mRNA was used as an internal standard for equal loading in this and other similar studies. For TNF-R2 mRNA level, it was enhanced in cells treated with 500 U/mL TNF-α as early as 5 minutes, remained high even after 48 hours, and the maximum induction was observed at around 2 hours. Thus, the induction was time-dependent. On the other hand, the mRNA levels of TNF-R1 and β-actin were relatively constant throughout the study times. This indicates that TNF-α is specifically induced TNF-R2 mRNA expression in C6 cells.

Another point of interest was that the level of TNF-R1 was higher than that of TNF-R2 in the control cells (Fig. 4). This suggests that, similar to primary cultured

astrocytes (Dopp *et al.*, 1997), TNF-R1 was the predominant TNF-α receptor subtype in C6 cells.

3.1.2 The signaling systems mediating TNF-α-induced TNF-R2 expression in C6 cells

The above study indicated that TNF-R2 expression was selectively up-regulated by the addition of TNF-α, it was therefore interested to investigate what was the signal transduction mechanism mediating this action of TNF-α. In this study, the possible roles of the PKC and PKA pathways were concentrated, as both are common signaling pathways in many tissues (Padmaperuma *et al.*, 1996; Zablocka *et al.*, 1998).

3.1.2.1 The involvement of PKC in TNF-α-induced TNF-R2 expression in C6 cells

In this study, the effects of a PKC activator, phorbol 12-myristate 13-acetate (PMA) and a PKC inhibitor, 3-{1-[3-(amidinothio)prophl]-3-indolyl}-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione mehanesulfonate (Ro31), on the induction of TNF-R2 expression by TNF-α were investigated. These agents were found to be selective

activator and inhibitor of PKC in C6 cells (Tsang *et al.*, 1997).

First, different dosages, 0.0162, 0.162, 1.62, 16.2 and 162 nM of PMA were added to the cultures. The mRNA contents of TNF-R1, -R2 and β-actin were semi-quantified by RT-PCR. As shown in Fig. 5, 1.62 nM of PMA had the most pronounced effect on TNF-R2 mRNA expression, but 0.162 nM also produced a very similar stimulatory effect. As the concentrations of PMA increased from 1.62 to 16.2 and 162 nM, the mRNA expression declined then increased again. The reason of this maybe related to the non-specific action or down-regulation of high concentrations of PMA (Holtmann & Wallach, 1987). On the other hand, PMA, at all concentrations tested had no effects on the expression of TNF-R1 and β-actin. This result suggests that activation of PKC can increase TNF-R2 expression in C6 cells and that there is concentration dependence.

To ensure the stimulation observed with PMA was specific and that the action of TNF-α was mediated through PKC, the inhibitory effect of Ro31 on TNF-α induction of TNF-R2 was studied. The dosage effects of Ro31 in the presence and absence of TNF-α on the mRNA levels of, TNF-R1 and -R2 were shown in Fig. 6. C6 Cells were treated with 10, 50, 100, 200 and 500 nM Ro31 alone for 2 hours (Fig. 6b), or in the presence of TNF-α (100 U/mL) for an additional 2 hours, and the mRNA levels for both TNF-Rs and β-actin were semi- quantified by RT-PCR as described in

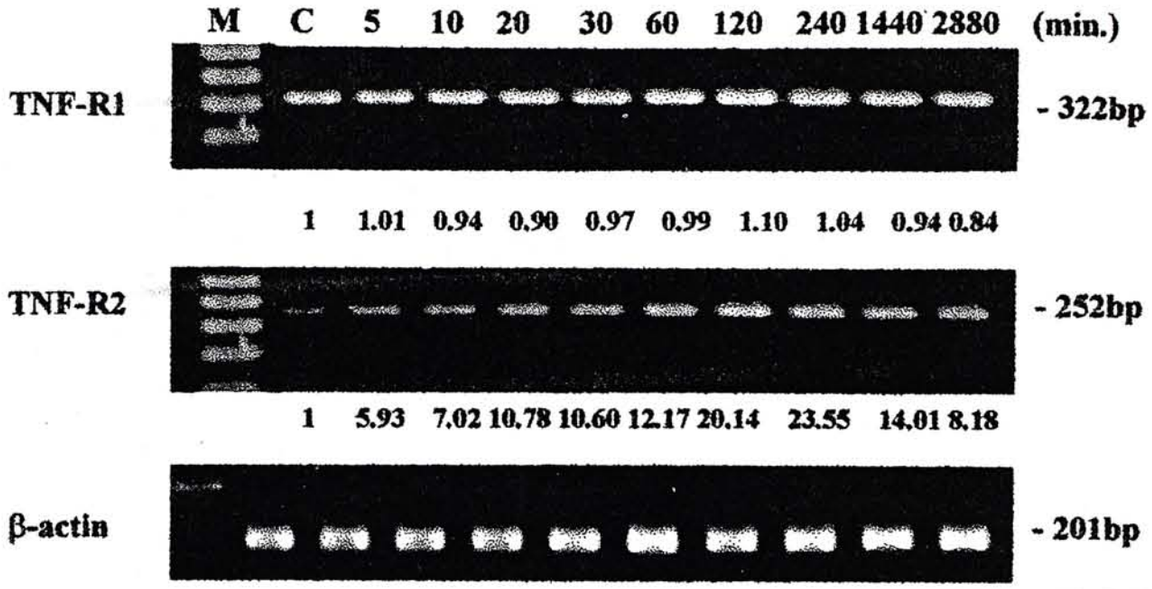

Fig. 4 Effect of TNF-α on the levels of TNF-R1, -R2 and β-actin mRNA in C6 cells. C6 Cells were treated with 500 U/mL TNF-α for 5 minutes to 48 hours and the total RNA was extracted and followed by RT-PCR as described in the Methods. After 30 cycles of amplification, a 5-μL aliquot of the PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed. The untreated cells (C) served as the control. The sizes, in bp, of the PCR products were indicated on the right. The DNA markers (M) were also run on the same gel. The amounts of the PCR products were semi-quantified by densitometry, and the value below each band represents the relative intensity after normalization with respect to that of β-actin. Data presented are representatives of three separate experiments with similar results.

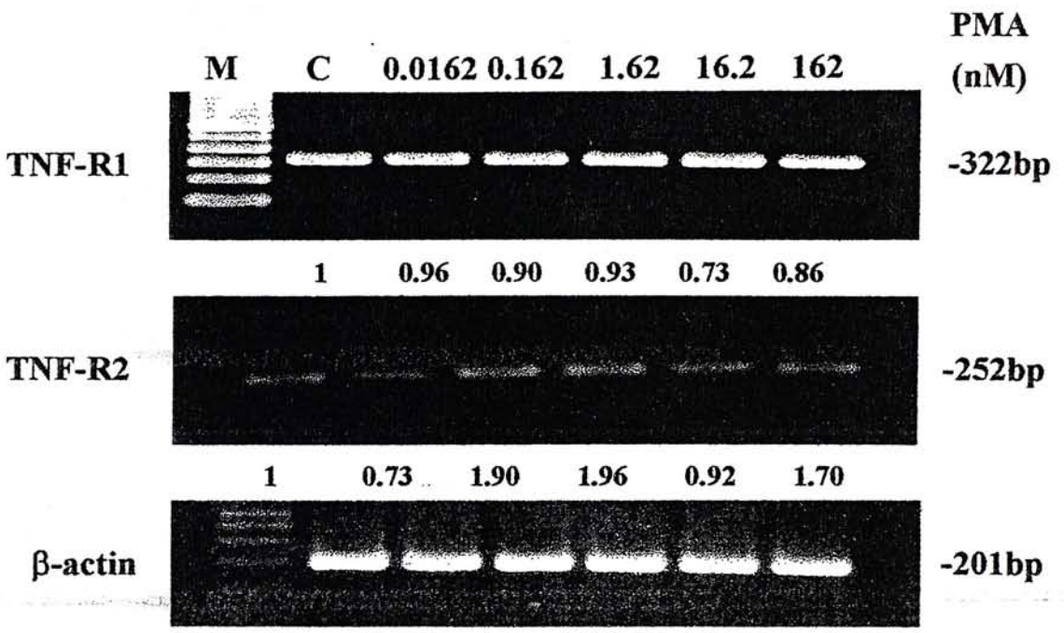

Fig. 5 Effect of PMA on the levels of TNF-R1, -R2 and β-actin mRNA in C6 cells. C6 Cells were treated with 0.0162 to 162 nM of PMA for 2 hours and the total RNA was extracted and followed by RT-PCR as described in the Methods. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

Fig. 4. Two hours pretreatment has been found to have an inhibition effectively (Tsang *et al.*, 1997). TNF-α at 100 U/mL had been found to produce maximum proliferative effect (Lung, 1999). Ro31 alone did not affect to any significant extent the expression of TNF-R2 mRNA at all concentrations of Ro31tested (Fig. 6b). However, the inhibitory action of Ro31 on the TNF-α induction of TNF-R2 was dose-dependent, and >50% inhibition were observed with 50 nM of Ro31, and a clear inhibition was observed with 10 nM of Ro31 (Fig. 6a). It should be noted that this low concentration of Ro31 alone did not decrease TNF-R2 level (Fig. 6b). It is also clear that Ro31 did not change levels of TNF-R1 mRNA much in the presence or absence of TNF-α (Figs. 6a and 6b). These results, together with the observation that PMA activated TNF-R2 expression (Fig. 5), suggest that PKC is involved in mediating the induction of TNF-R2 by TNF-α in C6 cells.

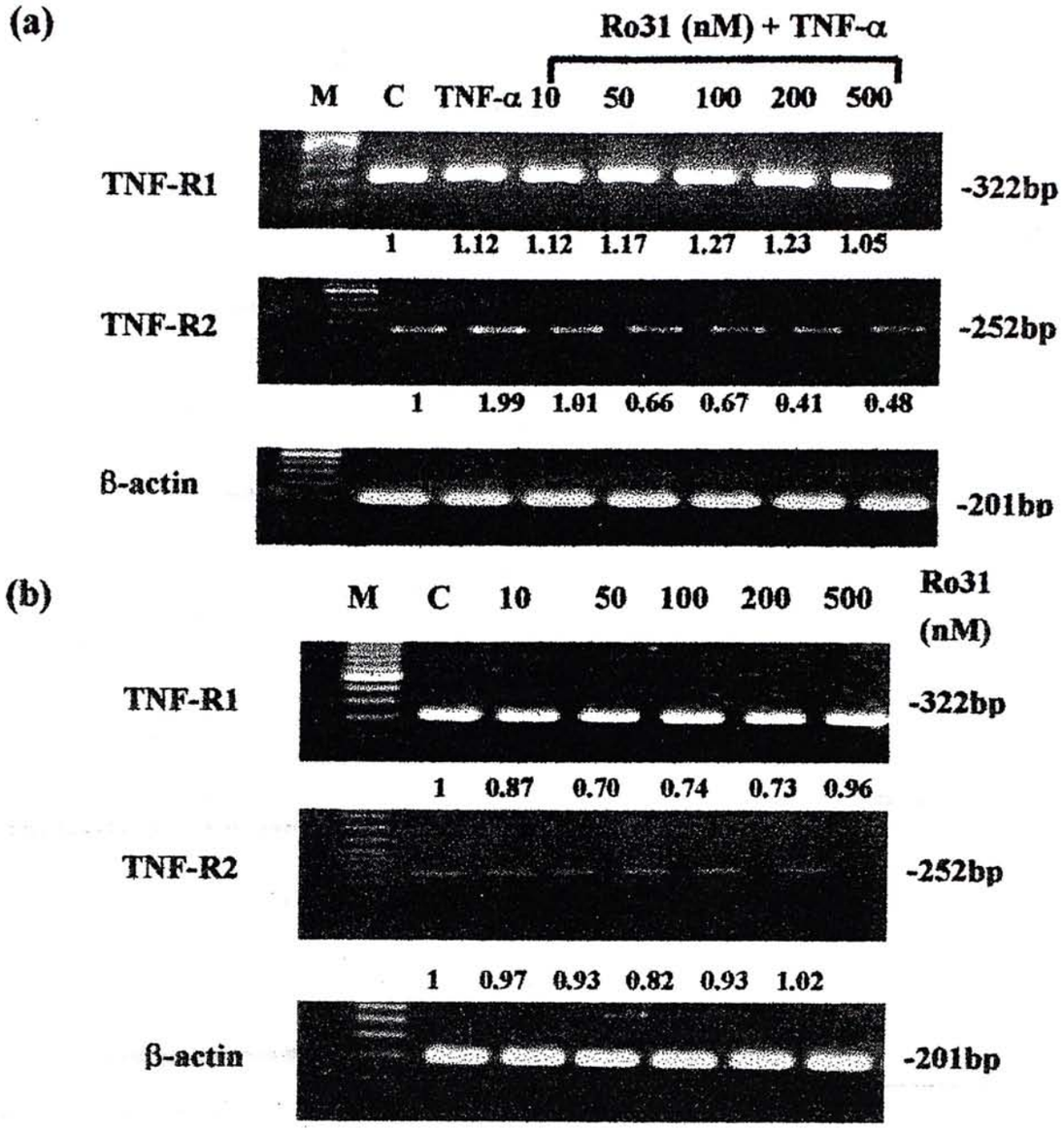

Fig. 6 Effect of Ro31 on the levels of TNF-R1, -R2 and β-actin mRNA in the presence or absence of TNF-α in C6 cells. (a) C6 Cells were treated with 100 U/ml TNF-α alone for 2 hours, or pre-treated with 10 to 500 nM Ro31 for 2 hours before the treatment with TNF-α. (b) C6 Cells were treated with 10 to 500 nM Ro31 only for 4 hours. The untreated cells were served as the control (C). The sizes, in bp, of the PCR bands were indicated on the right. Other details were as described as Fig. 4. Data presented are representatives of three separate experiments with similar results.

3.1.2.2 Effect of PMA on the TNF-R protein levels in C6 cells

To ensure that the TNF-R expression at the protein level was also affected in a manner similar to their respective mRNAs, the effect of PMA on both TNF-Rs was examined by Western blot. Fig. 7 showed the protein levels of the TNF-R1 and -R2 in C6 cells after exposure to PMA (1.62 nM) for 1 to 48 hours. This concentration was adopted as it produced maximum stimulation of TNF-R2 mRNA expression (Fig. 5). Two single bands of about 55 and 75 kDa were obtained, and the molecular masses of these bands were about the same as the protein products of the cloned TNF-R1 and R2 genes (Brockhaus *et al.*, 1990).

After exposure to PMA, the TNF-R2 protein was selectively induced at 24 hours and reached its optimum at 48 hours. These data indicated that this PKC activator could also up-regulate the protein level of TNF-R2 at 24 and 48 hours (Fig. 7) as well as that of mRNA (Fig. 5) and suggesting that the induction of both its mRNA and protein were coupled. The delay observed with protein expression maybe related to the time required for translation.

On the other hand, PMA, at all time points tested, inhibited the expression of TNF-R1 (Fig. 7). The inhibition or decrease in TNF-R1 by PMA was unclear at present and deserve further studies in the future.

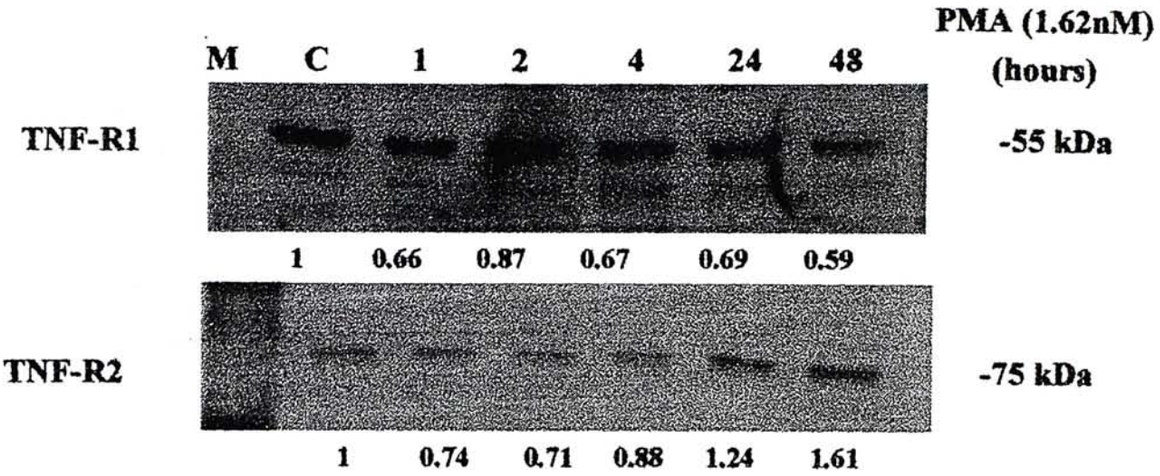

Fig. 7 Effect of PMA treatment on protein levels of TNF-R1 and -R2 in C6 cells. C6 Cells were treated with 1.62 nM PMA for 1, 2, 4, 24 and 48 hours. The untreated cell was served as the control (C). Thirty μg of membrane protein were electrophoresed in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane as described in the Methods. The protein bands of interest were detected using antibody against TNF-R1 or R2, and the luminescent signal generated was detected by exposing the membrane to an X-ray film. Protein markers (M) were also run on the same gel, and the sizes, in kDa, of protein bands were indicated on the right. The amount of the protein in each band was semi-quantified by densitometry, and the value below each band represents the relative intensity with reference to the respective controls (C). Data presented are representatives of three separate experiments with similar results.

3.1.2.3 Effect of Ro31 on the TNF-α-induced TNF-R2 protein level in C6 cells

As PMA induced the expression of TNF-R2 at the protein level, similar experiments were performed with Ro31 in the presence and absence of TNF-α. Fig. 8 showed the TNF-R2 protein level in C6 cells after exposure to Ro31 (200 nM) for different time intervals in the presence or absence of TNF-α (100 U/ml) for 4 hours. A single band of about 75 kDa was observed in the X-ray film, which is expected molecular size of TNF-R2 (Brockhaus *et al.*, 1990).

After exposure to Ro31, the TNF-α-induced TNF-R2 protein was suppressed when Ro31 was added 2 (decreased from 1.94 to 1.38) or 4 (decreased from 1.94 to 1.26) hours before the addition of TNF-α (Fig. 8). These data indicated that this PKC inhibitor could block the level of TNF-α-induced TNF-R2 protein. Treatment with Ro31 for 6 and 8 hours had no effect on TNF-R2 protein (Fig. 8). Similar experiment was also performed on TNF-R1, however, no effect on its expression was observed (data not shown).

Thus, PKC activator, PMA, enhanced the expressions of both TNF-R2 mRNA and protein, and that Ro31 suppressed the TNF-α-induced TNF-R2 mRNA and protein expression. These findings suggest that PKC is involved in mediating the induction of TNF-R2 in C6 cells.

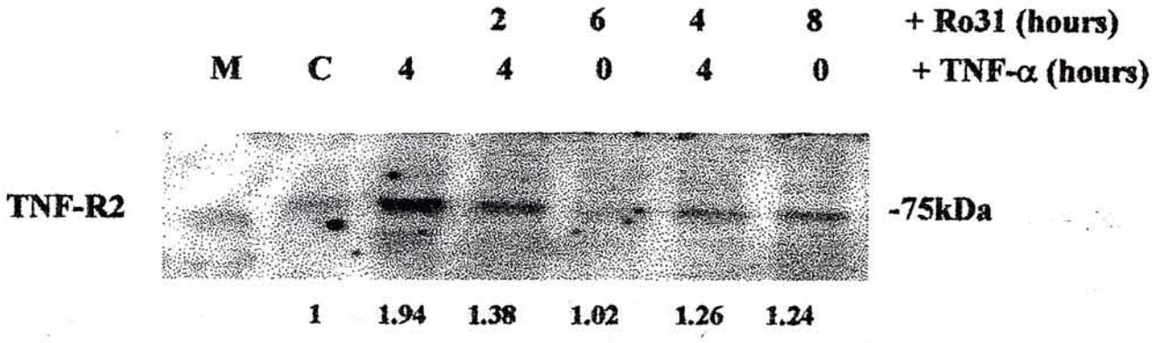

Fig. 8 Effect of Ro31 on the TNF-α-induced protein level of TNF-R2 in C6 cells. C6 Cells were treated with Ro31 (200 nM) with or without TNF-α (100 U/ml) for various time intervals as indicated. The untreated cells (C) served as the control. Other details were as described in Fig. 7. Data presented are representatives of three separate experiments with similar results.

3.1.2.4 Effect of PKA activator on the level of TNF-R2 mRNA in C6 cells

As a preliminary step to determine the specificity of PKC pathway in the TNF-α-induced TNF-R2 expression, the effect of a PKA activator, dbcAMP, on the TNF-R2 mRNA level in C6 cells was studied. Fig. 9 showed the effect of different dosages dbcAMP on TNF-R1, -R2 and β-actin mRNA levels. As can be seen, there was no clear effect of this PKA activator on both TNF-Rs mRNA expression. Though we had not tested the effect of any selective PKA inhibitors on the TNF-α-induced TNF-R expression, it is likely that PKA may not be a mediator in the induction of TNF-R2 in C6 cells. This is because dbcAMP is a very potent stimulator of PKA (Messens & Slegers, 1992). Other experiments are in progress in our laboratory to confirm this point and to determine the possible involvement of other signaling pathways in mediating the action of TNF-α on TNF-R2 expression.

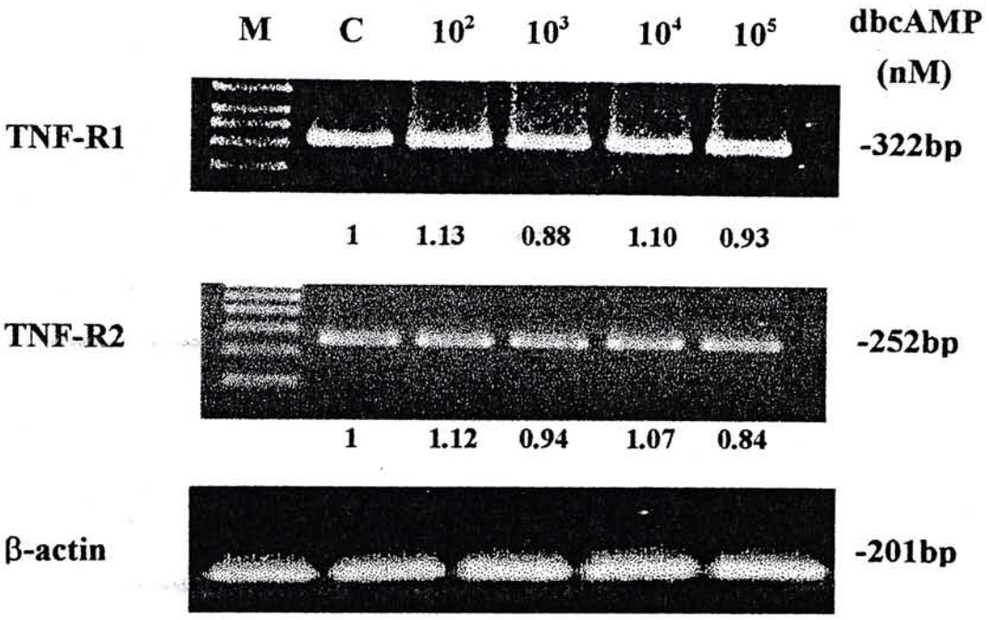

Fig. 9 Effect of various concentrations of dbcAMP on the levels of TNF-R1, -R2 and β-actin mRNA in C6 cells. C6 Cells were treated with 10^2 to 10^5 nM dbcAMP for 2 hours and the total RNA was extracted and followed by RT-PCR as described in the Methods. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

3.2 Effect of TNF-α on the expression of β1- and β2-adrenergic receptors (β1- and β2-ARs) in C6 glioma cells

As discussed in the Introduction, β-adrenergic mechanism, besides TNF-α, has been found to regulate astrocyte proliferation or astrogliosis (Hodges-Savola *et al.*, 1996; Sutin & Griffith, 1993). In our laboratory, Lung (1999) found that both TNF-α and isoproterenol, an β-AR agonist, induced C6 cell proliferation, so, it is likely that be a relationship exists between TNF-α and β-ARs. Thus, we investigated the effect of TNF-α on the expression of β1- and β2-AR genes in order to determine which receptor subtype(s) is/are responsible for the TNF-α-induced C6 cell proliferation. β3-AR was not included in this study as this receptor subtype is mainly expressed in adipose tissues (Strosberg, 1995).

To determine the effect of TNF-α on the β-AR expression in C6 cells, their mRNA levels were measured by RT-PCR, and their protein levels by Western blot analysis. Moreover, the signal transduction pathways mediating TNF-α-induced β-AR gene expression were also examined.

3.2.1 Effect of TNF-α on β1- and β2-ARs mRNA expression in C6 cells

A time-course experiment on the effect of TNF-α on β-ARs expression was performed. Fig. 10 showed the mRNA levels of β1- and β2-AR in C6 cells after exposure to TNF-α (100 U/ml) for different time intervals. This concentration of TNF-α was chosen because it induced maximum proliferation in C6 cells (Lung, 1999). Both β1- and β2-AR mRNA expression were enhanced in cells treated with 100 U/ml TNF-α from 5 minutes to 48 hours, and the maximum induction for β1- and β2-AR were observed at 30 and 60 minutes, respectively, and then reduced. This indicates that both β1- and β2-AR were induced by TNF-α, and that the response of β1-AR expression was much enhanced at 5 min. (5.4-fold increase) than that of β2-AR (2.54-fold). Moreover, both β-ARs were expressed in the untreated cells (Fig. 10).

3.2.2 The signaling systems mediating TNF-α-induced β1- and β2-AR expression in C6 cells

The above study (Fig. 10) showed that both β1- and β2-AR expression can be up-regulated by TNF-α, we therefore tried to investigate the signaling mechanism mediating this event. In this study, the possibility of PKC and PKA pathways were

concentrated, as they are common signaling pathways in many cell types.

3.2.2.1 The involvement of PKC mechanism between TNF-α and β-ARs in C6 cells

In this study, the effects of PMA and Ro-31 on the induction of β1- and β2-AR expression in the presence and absence of TNF-α were investigated.

Firstly, the effects of 0.0162, 0.162, 1.62, 16.2 and 162 nM of PMA, a PKC activator, on the mRNA levels of β1- and β2-AR were studied. The β1- and β2-AR mRNA contents were semi-quantified by RT-PCR as described in the Methods. As shown in Fig. 11, 1.62 nM of PMA produced the most pronounced stimulatory effect on both β1- and β2-AR mRNA expression and higher concentrations of PMA were inhibitory. This concentration was also most effective in stimulating TNF-R2 expression (Fig. 5). This suggests that PKC activation can induce both β-AR mRNA expressions. Two other points worthy of mentioning were: (a) β2-AR expression appeared to be more sensitive, as 0.162nM of PMA already slightly enhanced its expression by ~26%; and (b) that β1-AR expression was very much elevated (~10-fold) with the addition of 1.62nM PMA. The reason for the differential sensitivities is unclear.

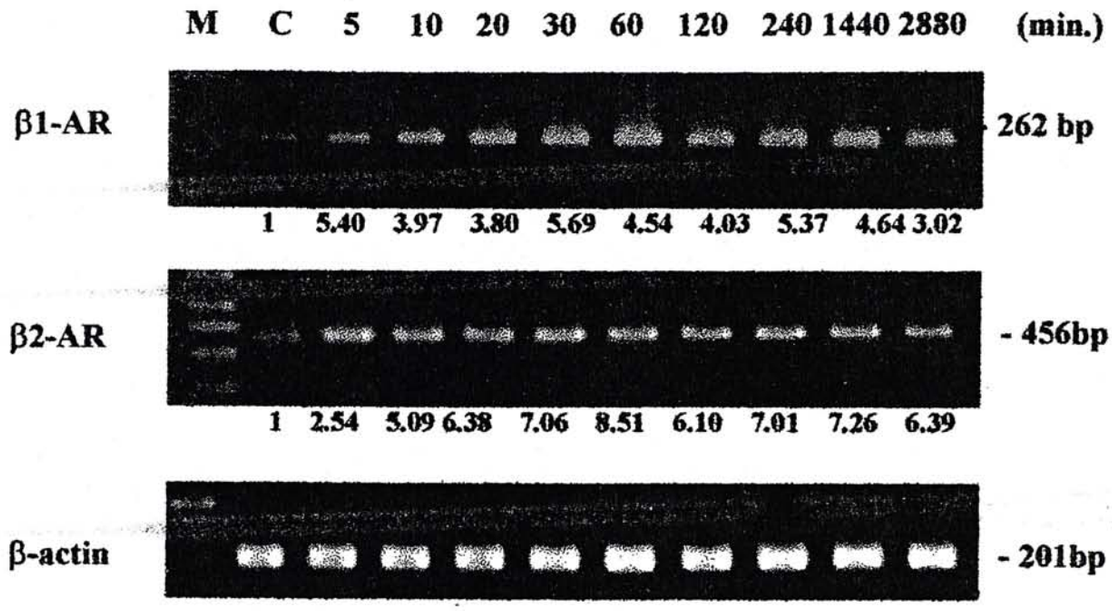

Fig. 10 Time course of exposure to TNF-α on the levels of β1-, β2-AR and β-actin mRNA in C6 cells. C6 Cells were treated with 100 U/ml TNF-α from 5 minutes to 48 hours and the total RNA was extracted and followed by RT-PCR as described in the Methods. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

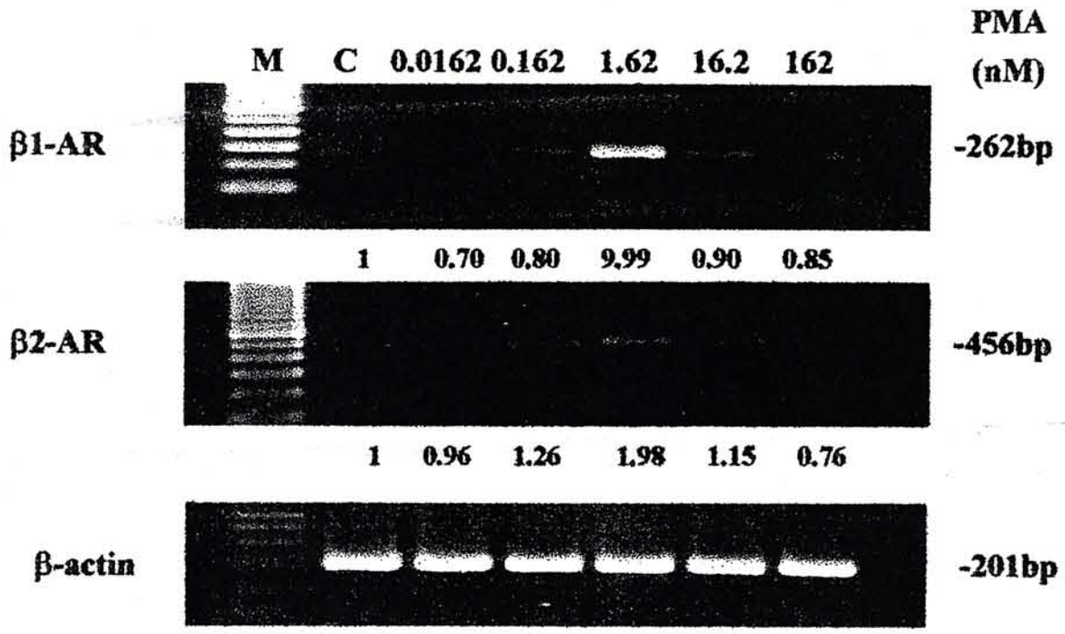

Fig. 11 Effect of various concentrations of PMA on the levels of β1-, β2-AR and β-actin mRNA in C6 cells. C6 Cells were treated with 0.0162 to 162 nM of PMA for 2 hours. The untreated cells (C) served as the control. Other details were described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

Secondly, the dosage effect of Ro31 on the TNF-α-induced mRNA levels of both β1- and β2-AR were studied (Fig. 12a). C6 Cells were treated with 10, 50, 100, 200 and 500 nM Ro31 for 2 hours before exposure to TNF-α for 2 hours, and the mRNA levels for both β-ARs and β-actin were semi-quantified by RT-PCR. The results showed that Ro31 at a concentration as low as 10 nM greatly reduced (≥50%) the expression of both β-ARs, and that this inhibitory effect was dose-dependent. Moreover, Ro31 alone at all concentrations tested, did not alter the expression of both β-AR (Fig. 12b). These results suggest that PKC is involved in the TNF-α-induced β1- and β2-AR gene expression in C6 cells.

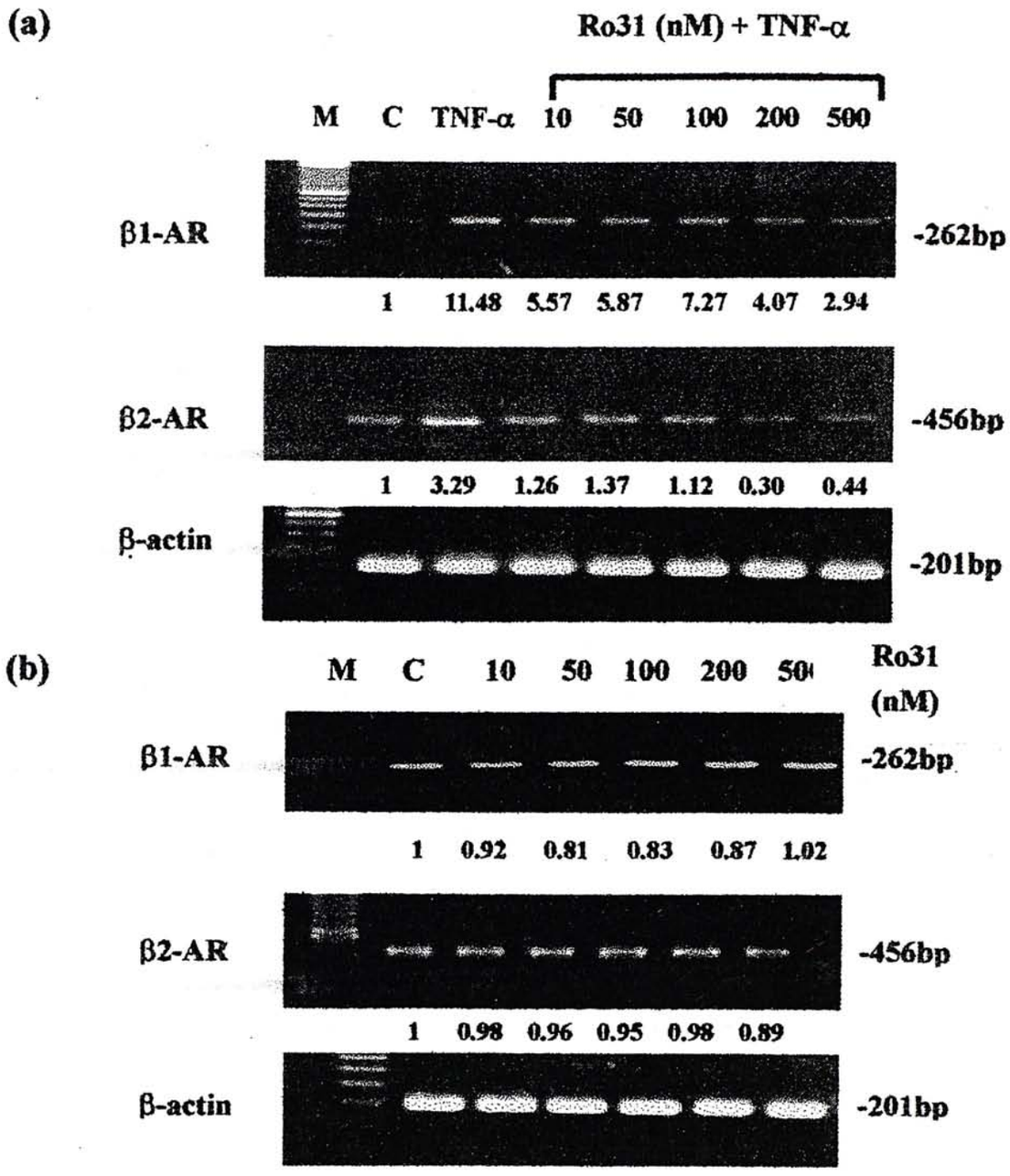

Fig. 12 Effect of various concentrations of Ro31 on the levels of β1-, β2-AR and β-actin mRNA in the presence and absence of TNF-α in C6 cells. (a) C6 Cells were treated with 100 U/ml TNF-α for 2 hours with or without pre-treated with Ro31 (10 to 500 nM) for 2 hours. (b) C6 Cells were treated with 10 to 500 nM Ro31 for 4 hours. The untreated cells (C) served as the control. The sizes, in bp, of the PCR bands were indicated on the right. Other details were as described in Fig. 11. Data presented are representatives of three separate experiments with similar results.

3.2.2.2 Effect of PMA on the β1- and β2-ARs protein levels in C6 cells

In order to examine whether the protein levels of both β-AR were affected in a similar manner as their mRNAs, the expression of both β-AR proteins were analysed by Western blotting following various treatments. Fig. 13 showed the protein levels of β1 and β-ARs in C6 cells after exposure to PMA (1.62 nM) for different time intervals. This concentration of PMA was chosen as it produced almost maximal effect on mRNA expression (Fig. 11). Two single bands of about 50 and 47 kDa, corresponding to β1- and β2-AR, respectively (Gocayne *et al.*, 1987: Buckland *et al.*, 1990; Machida *et al.*, 1990), were obtained on the X-ray films.. Comparing the densities of the two controls, the protein level of β2-AR was lower than that of β1-AR, and this indicates that β1-AR is the dominant receptor subtype in C6 cells.

After exposure to PMA, both β1- and β2-AR proteins were induced in 2 hours, and an optimum effect was observed at 48 hours. These data indicate that this PKC activator can up-regulate the protein levels of both β-AR.

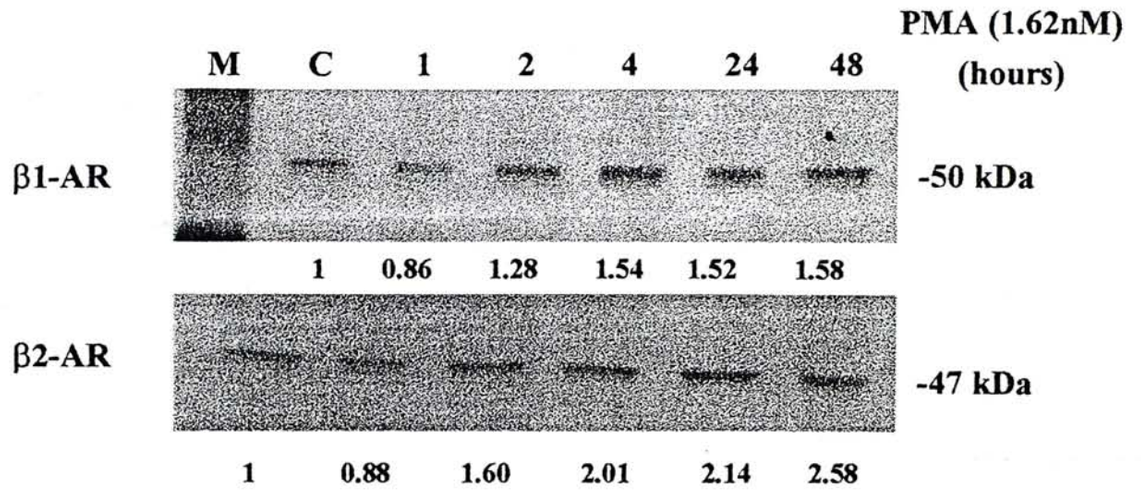

Fig. 13 Time course of PMA treatment on the protein levels of β1- and β2-AR in C6 cells. C6 Cells were treated with 1.62 nM PMA for 1, 2, 4, 24 and 48 hours. The untreated cells (C) served as the control. Thirty μg of membrane protein were electrophoresed in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. Protein bands of interest were detected by using antibody against β1- and β2-AR, respectively, and the luminescent signal generated was detected by exposing the membrane to an X-ray film. The protein markers (M) were also run on the same gel. The sizes, in kDa, of protein bands were indicated on the right. The amount of the protein in each band was semi-quantified by densitometry, and the value below each band represents the relative intensity with reference to the corresponding control. Data presented are representatives of three separate experiments with similar results.

3.2.2.3 Effect of Ro31 on the TNF-α-induced β1- and β2-AR protein levels in C6 cells

Fig. 14 showed the protein levels of the two β-ARs in C6 cells after exposure to Ro31 (200 nM) for different time intervals in the presence or absence of TNF-α (100 U/ml) for 4 hours. Two single bands of about 50 and 47 kDa, corresponding to the molecular weight of β1- and β2-ARs, were obtained on the X-ray films.

After exposure to Ro31 for various times, both the TNF-α-induced β1- and β2-AR proteins were suppressed. It was also clear that the longer the exposure time to Ro31 before TNF-α treatment, the greater was the inhibition (Fig. 14). These data indicate that this PKC inhibitor could block both TNF-α-induced β-AR protein as well as the mRNA (Fig. 12) levels. Ro31 alone, at 6 hours, did not change the levels of both β-ARs.

As PMA enhanced the protein expression of both β1- and β2-AR, and that Ro31 suppressed the TNF-α-induced β1- and β2-AR protein inductions, it suggests that PKC is involved in mediating the induction of both TNF-α-induced β1- and β2-AR in C6 cells.

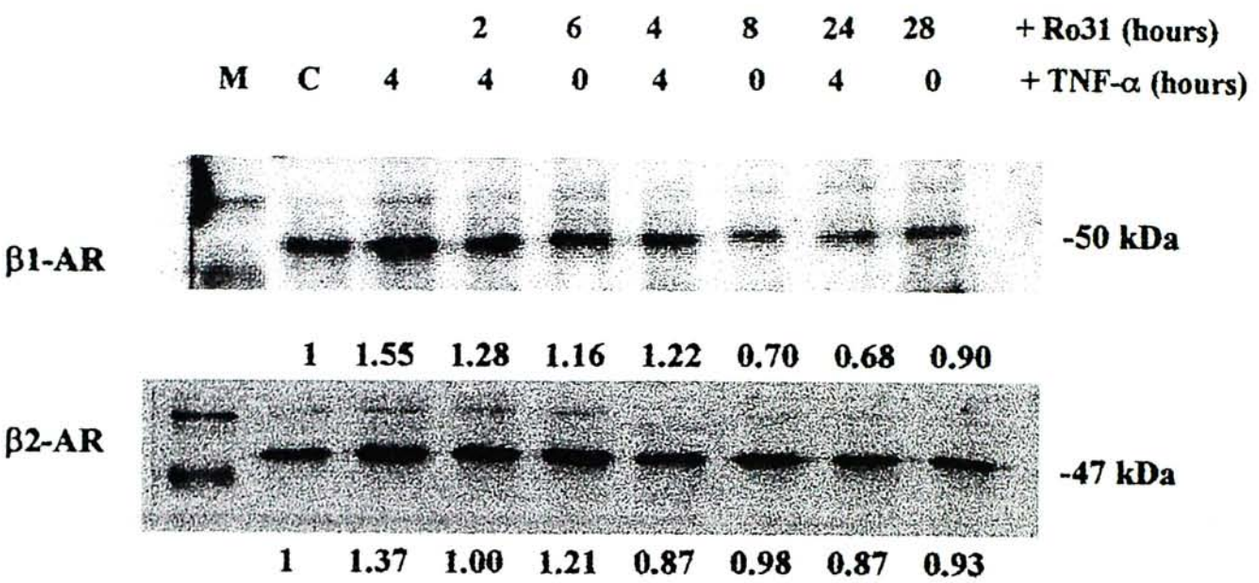

Fig. 14 Effect of Ro31 and TNF-α treatments on the protein levels of β1- and β2-AR in C6 cells. C6 Cells were treated with Ro31 (200 nM) in the presence or absence of TNF-α (100 U/ml) for various time intervals as indicated. The untreated cells (C) served as the control. Other details were described in Fig. 13. Data presented are representatives of three separate experiments with similar results.

3.2.2.4 Effect of dbcAMP on the levels of β1- and β2-ARs mRNA in C6 cells

To ensure the specificity of PKC pathway, the effect of a PKA activator, dbcAMP, on the β1- and β2-AR mRNA levels in C6 cells were studied. Figure 15 showed the effects of different doses of dbcAMP on β1-, β2-AR and β-actin mRNA levels. As can be seen, there was no clear effect of various concentrations of dbcAMP on both β1- and β2-AR mRNA expression. It is therefore suggested that PKA may not be the mediator in the induction of β1- and β2-ARs in C6 cells. As PMA induced, while Ro31 inhibited the induction of these proteins and mRNAs by TNF-α, it is very likely that one of the major second messengers mediating the induction is PKC. The possible involvement of other messengers is currently under investigation in our laboratory.

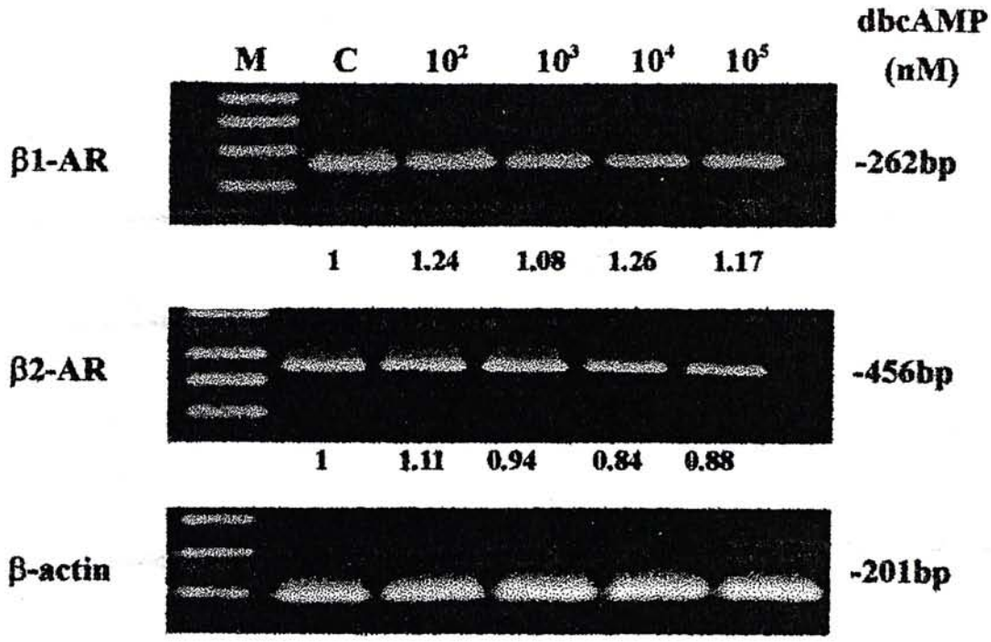

Fig. 15 Effect of various concentrations of dbcAMP on the levels of β1-, β2-ARs and β-actin mRNA in C6 cells. C6 Cells were treated with 10^2 to 10^5 nM dbcAMP for 2 hours, and the mRNA expression monitored by RT-PCR. The untreated cells (C) served as the control. Other details were described in Fig. 4. Data presented are representations of three separate experiments with similar results.

3.3 Relationship between TNF-R2 and β-adrenergic mechansim in C6 cells

As β-ARs have been found to regulate astrocyte proliferation (Liu, 1996) and astrogliosis (Hodges-Savola *et al.*, 1996; Sutin & Griffith, 1993), and that Lung (1999) in our laboratory had found isoproterenol induced proliferation in C6 cells, it is quite clear that β-adrenergic mechanism participates in C6 cell proliferation. Results presented in the previous sections showed that TNF-α induced both β-AR expression via its activation of TNF-R2 expression in C6 cells. In this section, we examined whether β-adrenergic mechanism had any effect on endogenous TNF-α and TNF-R2 expression by RT-PCR technique as described in **Section 2.4**.

3.3.1 Effects of isoproterenol and propranolol on endogenous TNF-α mRNA levels in C6 cells.

C6 cells were exposed to different dosages (0.01 to 10 μM) of isoproterenol for 2 hours, and the endogenous TNF-α mRNA levels were semi-quantified by RT-PCR (Fig. 16a). We observed that TNF-α was not expressed in the unstimuatled cells. The expression of TNF-α mRNA was enhanced by concentrations of isoproterenol greater than 0.1 μM, and a 10-fold stimulation was observed with 10 μM. This

stimulation was similar to that with TNF-α (100 U/ml) (Fig. 16a). To ensure the specificity of involvement of β-adrenergic mechanism, the effect of an β-antagonist, propranolol, in the suppression of TNF-α-induced endogenous TNF-α expression was examined. A clear inhibition was observed with 0.5 μM of propranolol, and there was concentration dependent with an optimum effect at 25 μM (Fig. 16a). Interestingly, the latter concentration of propranolol was found to be most effective in reducing TNF-α-induced proliferation in C6 cells (Liu, 1996). Endogenous TNF-α mRNA expression was not detected when C6 cells were treated with propranolol at all concentrations tested (Fig. 16b). This suggests that the β-adrenergic agonist exerts a positive regulation on endogenous TNF-α expression in C6 cells.

3.3.2 Effects of isoproterenol and propranolol on TNF-Rs mRNA levels in C6 cells

In this study, C6 cells were exposed to different dosages (0.01 to 10 μM) of isoproterenol for 2 hours, and the levels of both TNF-Rs mRNA expression were semi-quantified by RT-PCR (Fig. 17). The expression of TNF-R2 mRNA was enhanced by all the concentrations of isoproterenol tested. The increases were likely to be dose-dependent despite the relative intensity value with 1 μM was slightly lower than that with 0.1 μM. Although the stimulation by isoproterenol was lower than that

with TNF-α (100 U/ml); nevertheless, an enhancement of TNF-R2 expression of greater than five-fold was observed with 10 μM isoproterenol. Consistent with the involvement of an β-adrenergic mechanism, β-antagonist, propranolol, suppressed the TNF-α-induced TNF-R2 expression, and a clear inhibition was observed with 0.5 μM of propranolol, with an optimum effect at 25 μM (Fig. 17a). Interestingly, the latter concentration of propranolol was found to be most effective in reducing TNF-α-induced proliferation in C6 cells (Liu, 1996) as well as TNF-α-induced TNF-α mRNA level (Fig. 16). Propranolol alone, especially at low concentrations, did not have any clear effect on TNF-R2 mRNA expression (Fig. 17b). As isoproterenol did not affect TNF-R1 expression nor did propranolol in the presence or absence of TNF-α, these suggest that the β-adrenergic mechanism exert specific regulation on TNF-R2 expression in C6 cells.

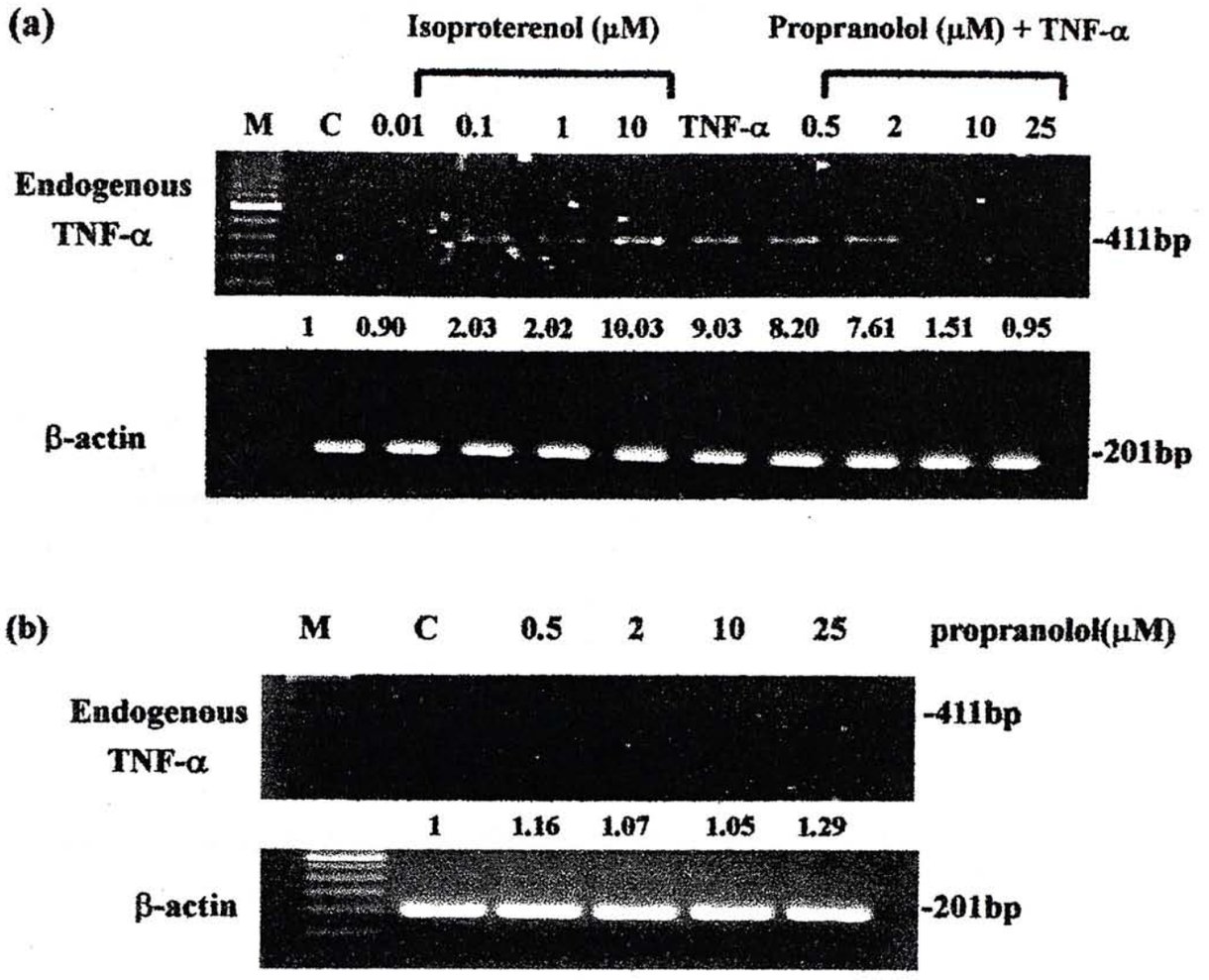

Fig. 16 Effects of various concentrations of isoproterenol and propranolol on the levels on endogenous TNF-α and β-actin mRNA in C6 cells. (a) C6 Cells were treated with 0.01 to 10 μM isoproterenol or 100 U/ml TNF-α for 2 hours, or pre-treated with propranolol (0.5 to 25 μM) for 2 hours before exposed to 100 U/ml TNF-α for another 2 hours. After 35 cycles of amplification, a 5-μL aliquot of the PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

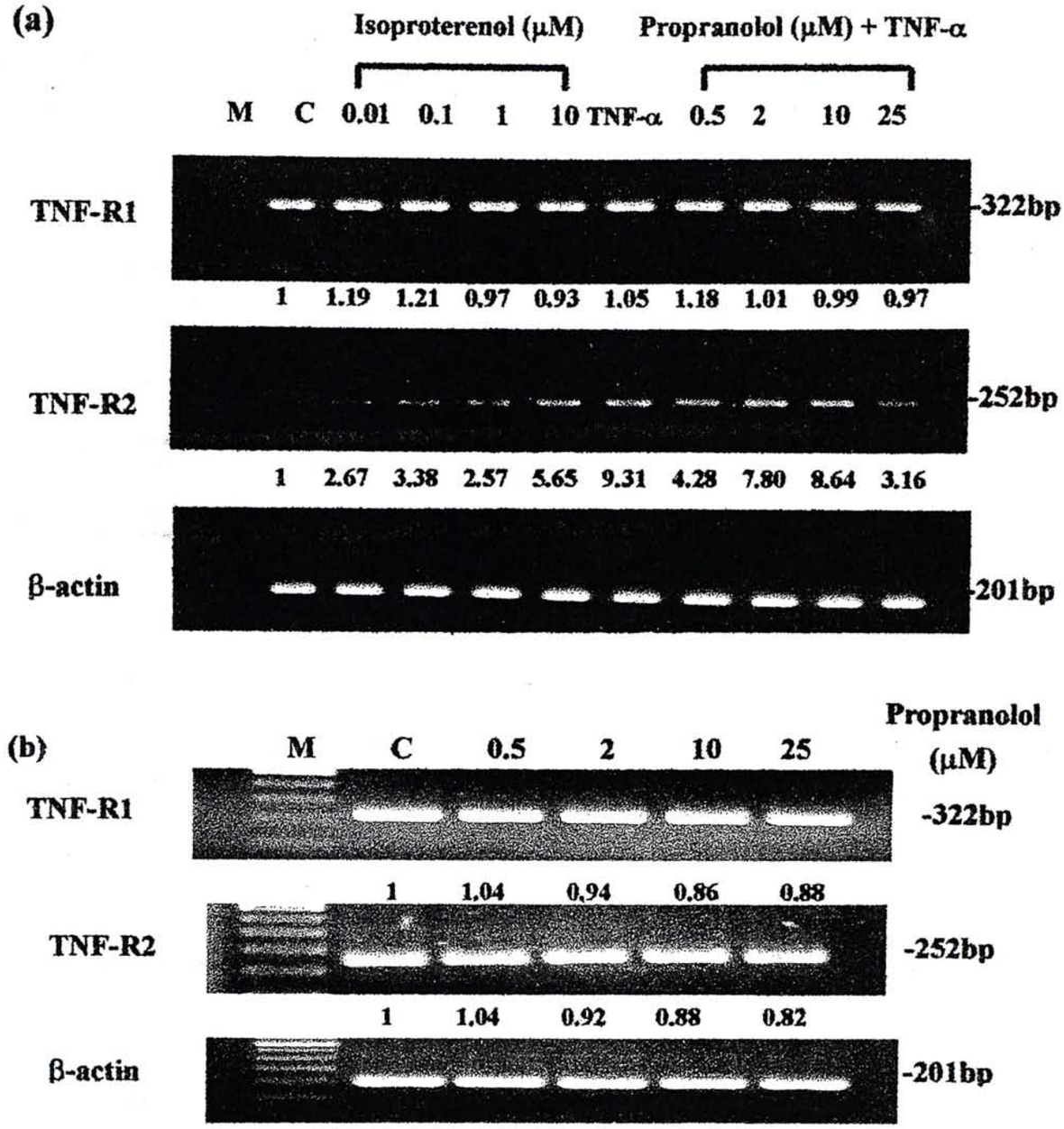

Fig. 17 Effects of various concentrations of isoproterenol and propranolol on the levels on TNF-R1, -R2 and β-actin mRNA in C6 cells. (a) C6 Cells were treated with 0.01 to 10 μM isoproterenol or 100 U/ml TNF-α for 2 hours, or pre-treated with propranolol (0.5 to 25 μM) for 2 hours before exposed to 100 U/ml TNF-α for another 2 hours. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

3.3.3 Effects of β1-agonist and antagonist on endogenous TNF-α mRNA expression in C6 cells

Since we observed that TNF-α induced the expression of both β1- and β2-AR (Fig. 10), we therefore examined the effects of some selective β-AR agonists and antagonists on endogenous TNF-α expression. The aim was to see whether these β-AR mechanisms play differential roles in modulating endogenous TNF-α expression. In this study, C6 cells were treated with 5, 10, 50, 100 and 500 nM of an β1-agonist, dobutamine (Deighton *et al.*, 1992) for 2 hours, and the mRNA levels of both endogenous TNF-α and β-actin were semi-quantified by RT-PCR. The TNF-α mRNA expression was not detected in untreated cells, but enhanced in a dose dependent manner in cells treated with different concentrations of dobutamine, and maximum induction was observed with 500 nM (Fig. 18). On the other hand, the expression of β-actin was relatively constant at all concentrations of dobutamine tested. This finding suggests that the activation of β1-AR would increase the expression of endogenous TNF-α expression in C6 cells.

To ensure the observations with dobutamine (Fig. 18) indeed reflected the involvement of the β1-adrenergic mechanism in regulating endogenous TNF-α expression in C6 cells, the dosage effects of an β1-antagonist, atenolol (Koganei *et al.*,

1995), in the presence or absence of TNF-α (100 U/mL), on the levels of endogeneous TNF-α mRNA were studied (Fig. 19). In this study, C6 cells were treated with 50, 100, 500 nM and 1 μM of atenolol for 2 hours then following by the addition of 100 U/ml TNF-α for another 2 hours, and the mRNA levels for endogenous TNF-α and β-actin were semi-quantified by RT-PCR. The TNF-α-induced endogenous TNF-α mRNA level were suppressed by the addition of atenolol (by 28% with 50 nM). Complete inhibition was observed with 1 μM of atenolol. However, endogenous TNF-α mRNA expression was not detected in untreated cells as well as cells treated with atenolol alone (Fig. 19b). This finding, taken together with the observation that dobutamine induced endogenous TNF-α, suggest that β1-adrenergic mechanism regulates the expression of endogenous TNF-α in C6 cells.

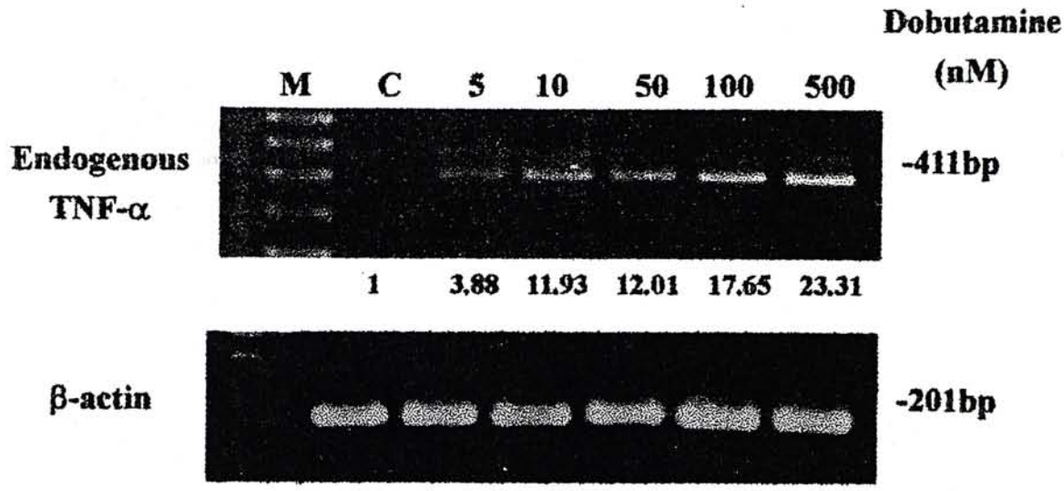

Fig. 18 Effect of various concentrations of dobutamine on the levels of endogenous TNF-α and β-actin mRNA in C6 cells. C6 Cells were treated with 5 to 500 nM of dobutamine for 2 hours, and the total RNA was extracted and followed by RT-PCR as described in the Methods. The untreated cells (C) served as the control. Other details were described in Fig. 16. Data presented are representatives of three separate experiments with similar results.

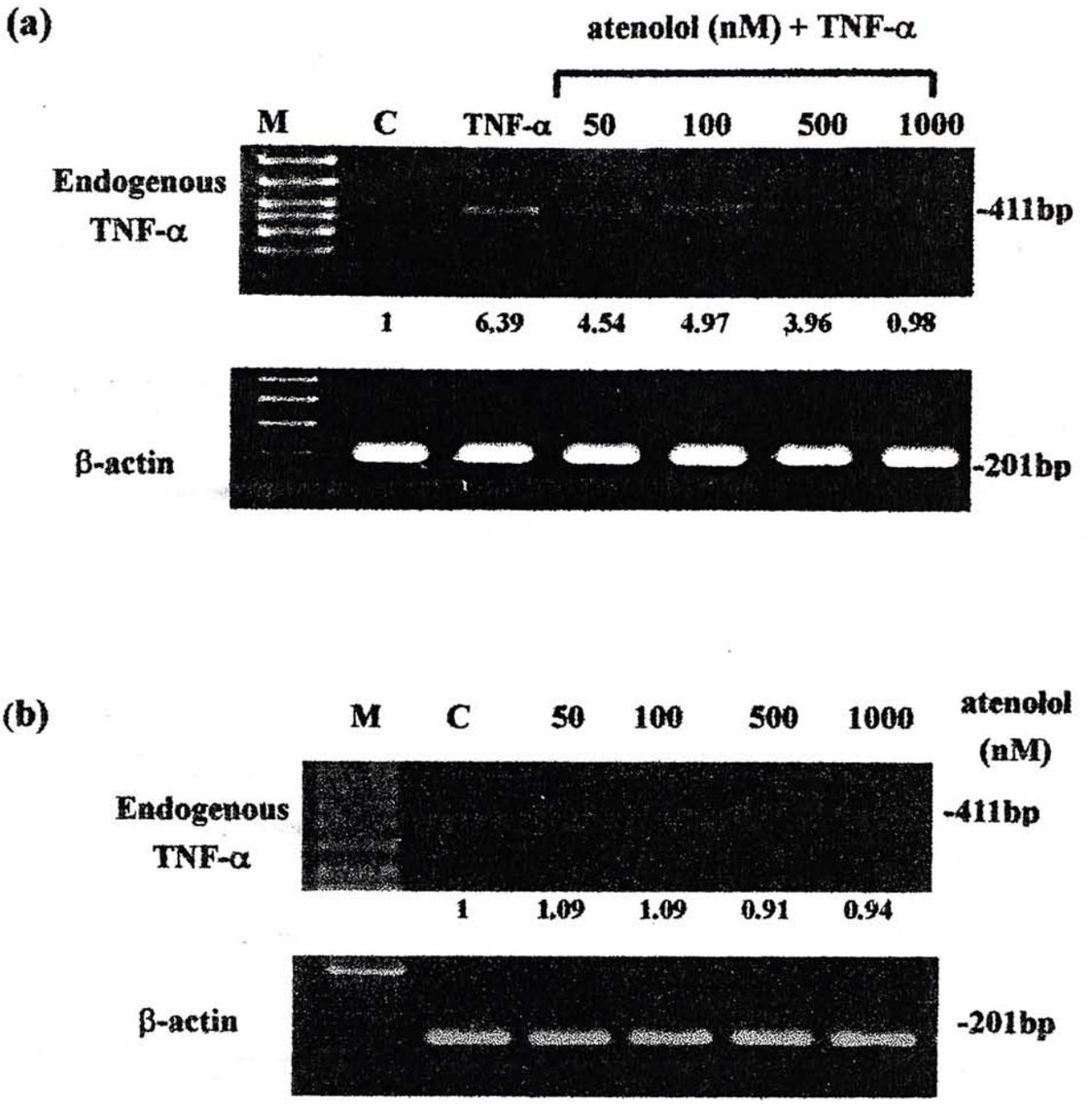

Fig. 19 Effect of various concentrations of atenolol on the levels of endogenous TNF-α and β-actin mRNA in TNF-α-treated C6 cells. (a) C6 Cells were pre-treated with 50 nM to 1 μM atenolol for 2 hours before exposed to TNF-α (100 U/ml) for another 2 hours. (b) C6 Cells were treated with 50 nM to 1 μM atenolol for 4 hours. The untreated cells (C) served as the control. Other details were as described in Fig. 16. Data presented are representatives of three separate experiments with similar results.

3.3.4 Effects of β1-agonist and antagonist on TNF-R2 mRNA expression in C6 cells

Since we observed that TNF-α induced the expression of both β1- and β2-AR (Fig. 10) and that dobutamine induced (Fig. 18) but atenolol reduced TNF-α-induced TNF-α expression (Fig. 19), we therefore examined the effects of dobutamine, a selective β1-AR agonist, and atenolol, an antagonist, on TNF-R2 expression. The aim was to see whether TNF-R2 expression was also affected. In the first study, cells were treated with different dosages of a selective β1-agonist, dobutamine (Deighton *et al.,* 1992), and its effect on TNF-R mRNA expression in C6 cells examined. Briefly, C6 cells were treated with 5, 10, 50, 100 and 500 nM dobutamine for 2 hours, and the mRNA levels of both TNF-Rs and β-actin were semi-quantified by RT-PCR. The TNF-R2 mRNA expression was enhanced in cells when treated with dobutamine, and a maximum induction (2.8 folds) was observed with 500 nM (Fig. 20). An 23-fold increase in TNF-α was observed with this concentration of dobutamine (Fig. 18). On the other hand, the expression of TNF-R1 and β-actin were relatively constant at all concentrations of dobutamine tested. This finding suggests that the activation of β1-AR can increase the expression of TNF-R2 expression in C6 cells.

Next, we examined the effect of exposure time to dobutamine (500 nM) on the

levels of TNF-R1 and -R2 mRNA expression in C6 cells. This concentration of dobutamine was chosen as it enhanced TNF-α expression by 23 folds (Fig. 18) and TNF-R2 expression by 2.8 folds (Fig. 20). An 2.4 folds increase in TNF-R2 mRNA was observed in cells treated with 0.5 μM dobutamine for 30 mins, and peaked at 120 minutes (4 folds), then declined (Fig. 21). No clear effects on TNF-R1 and β-actin expression were observed during the times studied (Fig. 21). This finding suggests that the activation of β1-AR increases the expression of TNF-R2 in C6 cells.

To ascertain that the observations with dobutamine (Figs. 20 and 21) indeed reflected the involvement of the β1-adrenergic mechanism in regulating TNF-R2 expression in C6 cells. The dosage effects of a selective β1-antagonist, atenolol (Koganei *et al.*, 1995), in the presence or absence of TNF-α (100 U/ml) on the expression of TNF-R2 mRNA were studied (Figs. 22). In this study, C6 cells were treated with 50, 100, 500 nM and 1 μM of atenolol for 2 hours then followed by the addition of 100 U/ml TNF-α for another 2 hours, and the mRNA levels for both TNF-Rs and β-actin were semi-quantified by RT-PCR. The TNF-α-induced TNF-R2 mRNA levels were greatly suppressed (~70% with 50 nM atenolol) by the addition of atenolol, while the levels of TNF-R1 and β-actin were relatively unaffected (Fig. 22a). However, atenolol alone, at all concentrations tested, did not cause any clear changes in the expression of TNF-R1, -R2 and β-actin mRNA at all concentrations tested (Fig.

22b). This finding, taken together with the observation that dobutamine induced TNF-R2 selectively, suggest that β1-adrenergic mechanism regulates the expression of TNF-R2 in C6 cells.

3.3.5 Effects of β2-agonist and antagonist on endogenous TNF-α mRNA in C6 cells

Besides β1-AR, we also studied the effects of β2-adrenergic mechanism on endogenous TNF-α mRNA expression. In this study, C6 cells were treated with 5, 10, 50, 100 and 500 nM of a selective β2-agonist, procaterol (Koganei *et al.*, 1995) for 2 hours, and the mRNA levels of both endogenous TNF-α and β-actin were semi-quantified by RT-PCR. The TNF-α mRNA induction was observed with 5 nM procaterol, and then slightly declined to almost the control level as the concentrations of procaterol increased. However, the levels of β-actin mRNA was relatively constant at all concentrations of procaterol tested (Fig. 23). This study suggests that β2-adrenergic mechanism regulates expression of endogenous TNF-α in C6 cells.

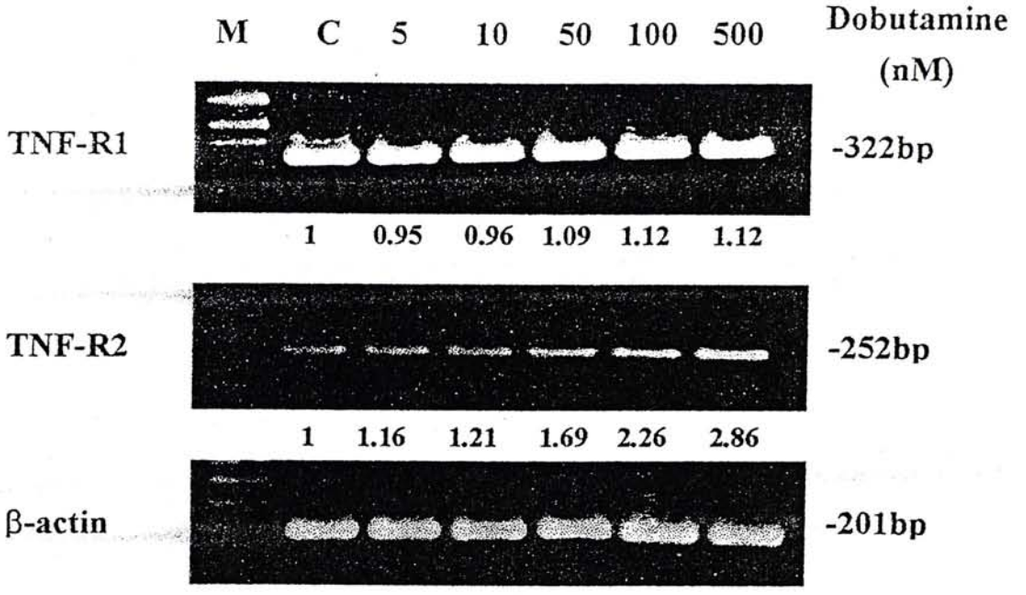

Fig. 20 Effect of various concentrations of dobutamine on the levels of TNF-R1, -R2 and β-actin mRNA in C6 cells. C6 Cells were treated with 5 to 500 nM of dobutamine for 2 hours, and the total RNA was extracted and followed by RT-PCR as described in the Methods. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

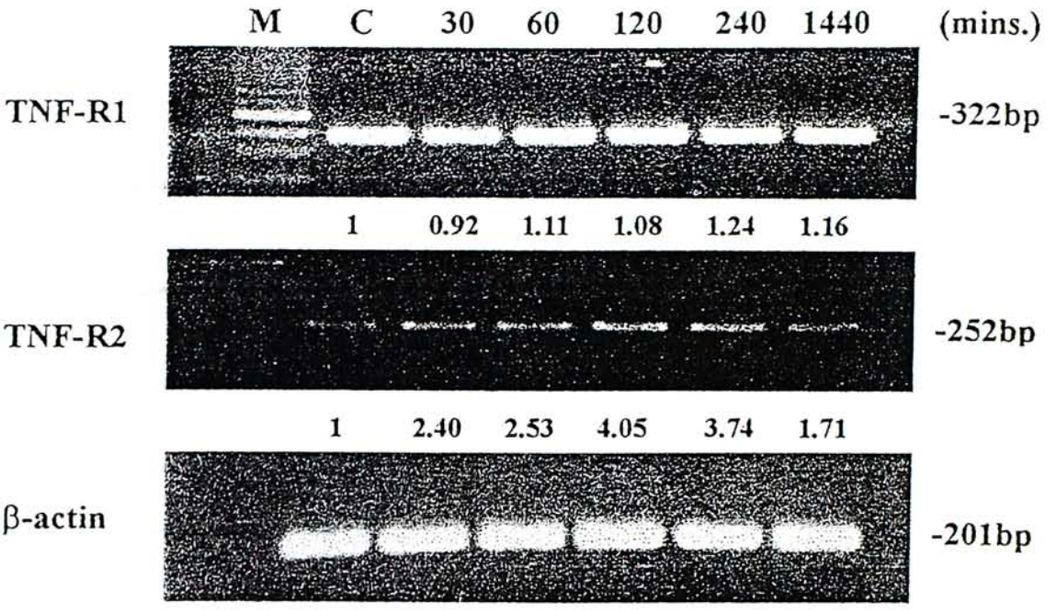

Fig. 21 Time course of dobutamine on TNF-R1, -R2 and β-actin mRNA expression in C6 cells. C6 Cells were treated with 500 nM dobutamine for 30 minutes to 24 hours, and the untreated cells (C) served as the control. The sizes, in bp, of the PCR products were indicated on the right. Other details were described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

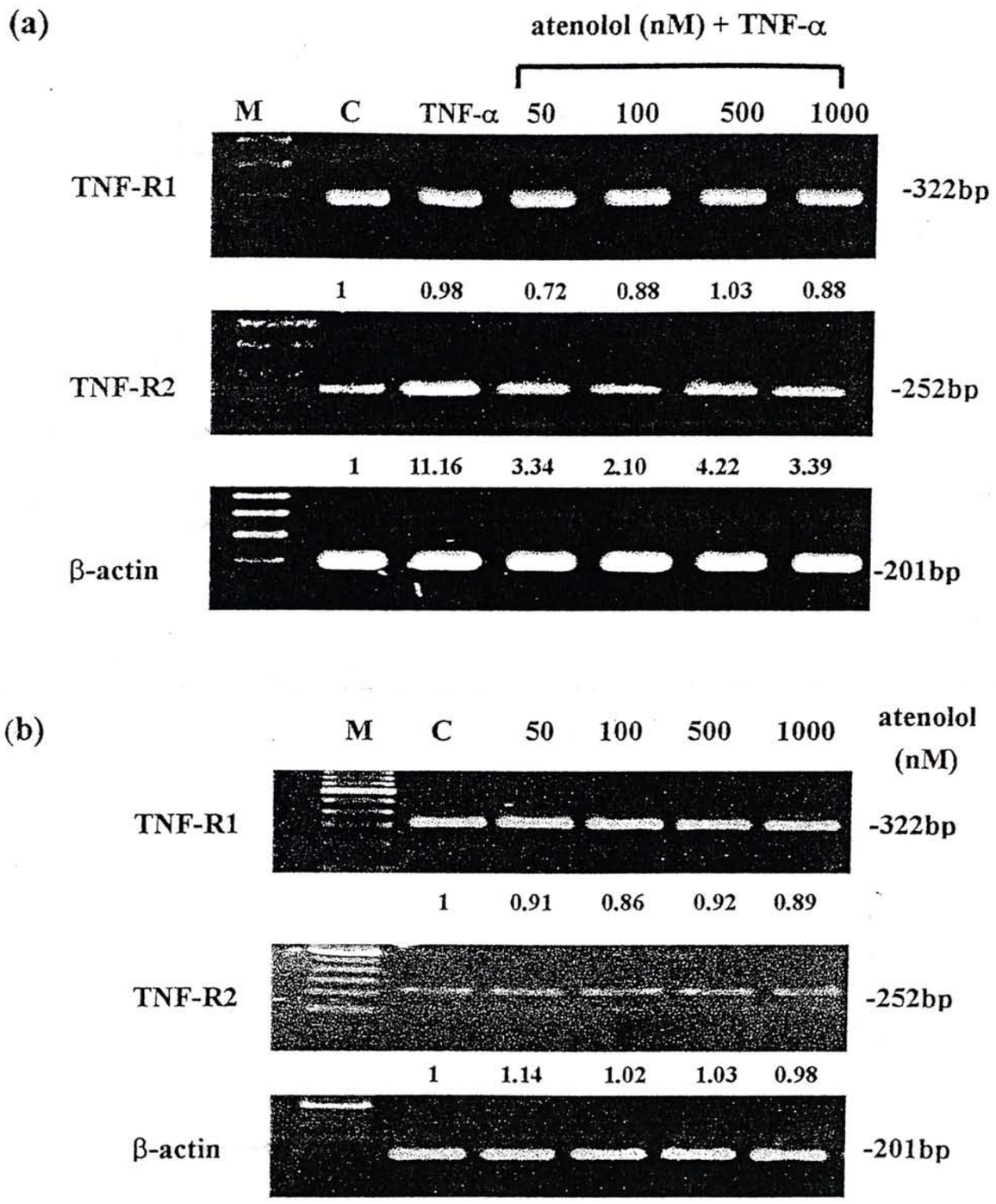

Fig. 22 Effect of various concentrations of atenolol on the levels of TNF-R1, -R2 and β-actin mRNA in TNF-α-treated C6 cells. (a) C6 Cells were pre-treated with 50 nM to 1 μM atenolol for 2 hours before exposed to TNF-α (100 U/ml) for another 2 hours. (b) C6 Cells were treated with 50 nM to 1 μM atenolol for 4 hours. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

To ensure the observations with procaterol (Fig. 23) indeed reflected the involvement of the β2-adrenergic mechanism in regulating endogenous TNF-α expression in C6 cells, the dosage effects of a selective β2-antagonist, ICI 118,551 (Deighton *et al.*, 1992), in the presence or absence of TNF-α (100 U/ml), on the levels of endogeneous TNF-α mRNA were studied (Fig. 24). In this study, C6 cells were treated with 10 nM, 50 nM, 0.1 μM and 0.5 μM of ICI 118,551 for 2 hours then followed by the addition of 100 U/ml TNF-α for another 2 hours, and the mRNA levels for endogenous TNF-α and β-actin were semi-quantified by RT-PCR. The TNF-α-induced endogenous TNF-α mRNA level were greatly suppressed by the addition of ICI 118,551. This antagonist, at 10 nM, reduced the TNF-α-induced endogenous TNF-α expression by ~48% and complete inhibition was observed with high concentrations (100 and 500 nM) of this compound (Fig. 24a). However, ICI 118,551 alone did not cause any clear changes in the expression of endogenous TNF-α and β-actin mRNA at all concentrations tested (Fig. 24b). This finding, taken together with the observation that procaterol induced endogenous TNF-α, suggests that β2-adrenergic mechanism, similar to that of β1-adrenergic, also regulates the expression of endogenous TNF-α in C6 cells.

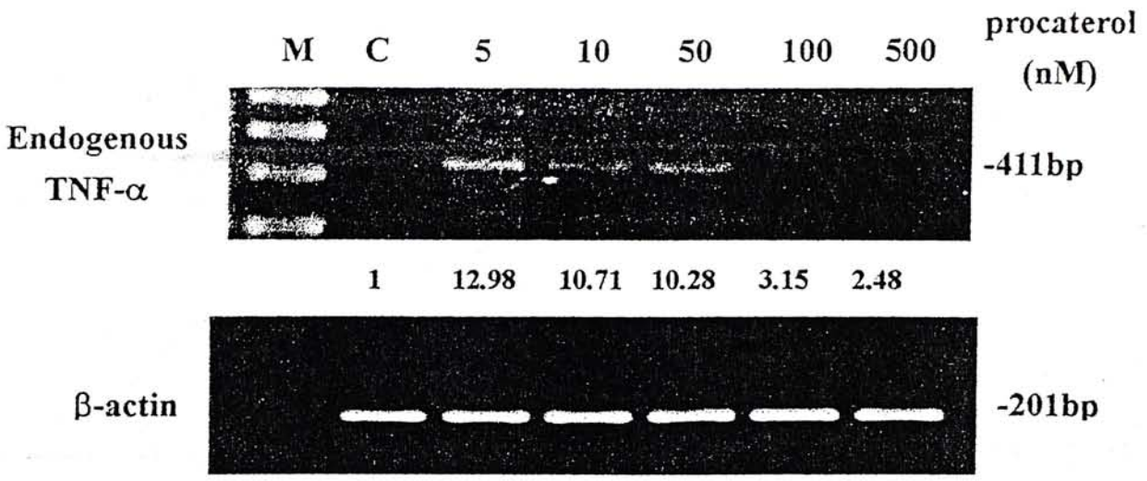

Fig. 23 Effect of various concentrations of procaterol on the levels of endogenous TNF-α and β-actin mRNA expression in C6 cells. C6 Cells were treated with 5 nM to 0.5 μM of procaterol for 2 hours and the total RNA was extracted and followed by RT-PCR as described in the Methods. The untreated cells (C) served as the control. Other details were as described in Fig. 16. Data presented are representatives of three separate experiments with similar results.

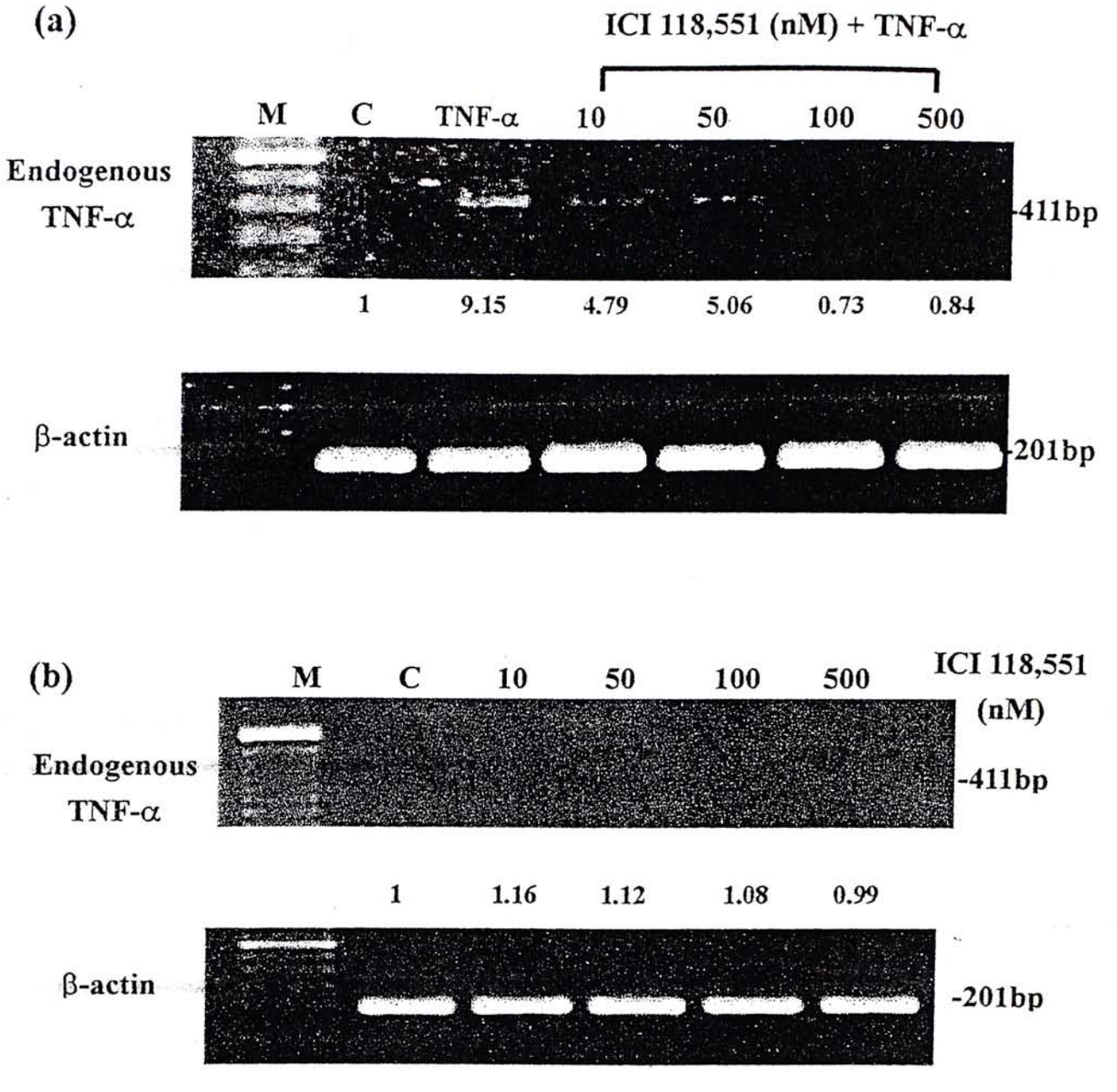

Fig. 24 Effect of various concentrations of ICI 118,551 on the levels of endogenous TNF-α and β-actin mRNA in TNF-α-treated C6 cells. (a) C6 Cells were treated with 100 U/ml TNF-α, or pre-treated with 10 nM to 0.5 μM ICI 118,551 for 2 hours, before the treatment of TNF-α for another 2 hours. (b) C6 Cells were treated with 10 nM to 1 μM ICI 118,551 for 4 hours. The untreated cells (C) served as the control. Other details were as described in Fig. 16. Data presented are representatives of three separate experiments with similar results.

3.3.6 Effects of β2-agonist and antagonist on TNF-R2 mRNA in C6 cells

As β2-AR expression was also induced by TNF-α (Fig. 10) and that β2-agonist stimulated TNF-α expression, while β2-antagonist suppressed TNF-α-induced TNF-α expression, it would be of interest to test whether selective β2-agonist and antagonist affect TNF-Rs expression. In this study, cells were treated with different dosages (5, 10, 50, 100 and 500 nM) of a selective β2-agonist, procaterol (Koganei *et al.*, 1995) for 2 hours, and its effect on the levels TNF-R1, -R2 and β-actin mRNA in C6 cells was measured by RT-PCR (Fig. 25). The TNF-R2 mRNA induction was observed with 5 to 50 nM procaterol (by 2.9 to 2.2 folds, respectively), and then declined to the control level as the concentrations of procaterol increased. However, the levels of TNF-R1 and β-actin mRNA were relatively constant at all concentrations of procaterol tested.

A time-course experiment on the effects of procaterol on both TNF-Rs and β-actin expression was also performed. Fig. 26 showed the mRNA levels of TNF-R1 and R2 after exposure to procaterol (10 nM) for different time intervals, and a maximum induction was observed at around 2 hour and remained relatively high even at 24 hours. On the other hand, the effect of this β2-agonist on TNF-R1 was rather small. These data suggest that β2-adrenergic mechanism regulates expression of TNF-

R2, in addition to TNF-α, in C6 cells.

To ascertain this stimulating result of procaterol, we investigated the effect of ICI 118, 551, a selective β2-antagonist (Deighton *et al.*, 1992) on TNF-α-induced TNF-R2 expression. In this study, C6 cells were treated with 10 nM, 50 nM, 0.1 μM and 0.5 μM of ICI 118,551 for 2 hours before the addition of 100 U/ml TNF-α for another 2 hours, and the mRNA levels for both TNF-Rs as well as that of β-actin were semi-quantified by RT-PCR. The TNF-α-induced TNF-R2 mRNA levels were suppressed by the addition of 10 and 50 nM of ICI 118,551 by 20% and 56%, respectively, and the maximum inhibition was observed with 500 nM (Fig. 27). However, ICI 118,551 also inhibited the expression of TNF-R1 slightly (Fig. 27). This β2-antagonist alone did not cause any significant changes in the expression of TNF-R1, -R2 and β-actin mRNA at all concentrations tested (Fig. 27b). These results suggest that β2-adrenergic mechanism also regulates the induction of TNF-R2 in C6 cells, but the effect is much less pronounced than that of β1-antagonist (Fig. 22). The observation that ICI 118, 551 reduced the expression of both TNF-Rs in the presence of TNF-α, but had no effect on the basal of these receptor subtypes is intriguing. The question whether this is due to the non-specific effect of this β2-antagonist remains to be examined. Another point of interest is that the stimulatory action of procaterol (an β2-agonist) was faster than that of dobutamine (an β1-agonist) (comparing Figs. 21

and 26). This would imply that the action of β2-adrenergic mechanism was faster, though less effective than that of β1.

3.4 Effects of TNF-α on the expression of a transcriptional factor nuclear factor kappa B (NF-κB) in C6 glioma cells

Results presented in the earlier sections suggest that TNF-α-induced C6 cell proliferation by activating TNF-R2 and β-ARs. However, the mechanism leading to proliferation in C6 cells is unclear. Since the induction of transcription factor(s) and/or early genes are commonly involved in cell proliferation, we therefore, examined the effect of TNF-α on NF-κB expression. NF-κB was found to be activated by several cytokines and neurotrophic factors in a number of cell types (Sullivan *et al.*, 1999), and particularly by TNF-α (Mattson *et al.*, 2000). Consistent with this idea is a recent report which stated that NF-κB had a wide range of functions: cytokine production, immune activation, and especially, proliferation and differentiation (Lim *et al.*, 2000). In the present study, the p50 subunit of NF-κB was measured by RT-PCR. This subunit will form a dimer with the p65 subunit and translocates to the nucleus and then binds selectively to genes responsive to NF-κB (Mattson *et al.*, 2000). Moreover, the signal transduction mechanism between TNF-α

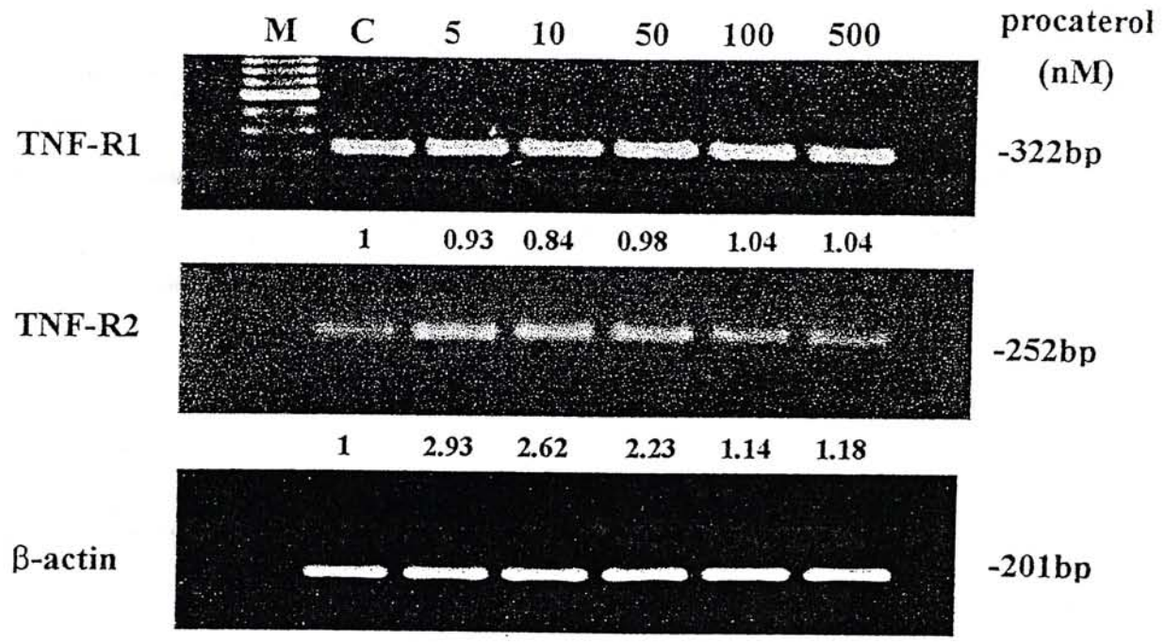

Fig. 25 Effect of various concentrations of procaterol on the levels of TNF-R1, -R2 and β-actin mRNA expression in C6 cells. C6 Cells were treated with 5 nM to 0.5 μM of procaterol for 2 hours and the total RNA was extracted and followed by RT-PCR as described in the Methods. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

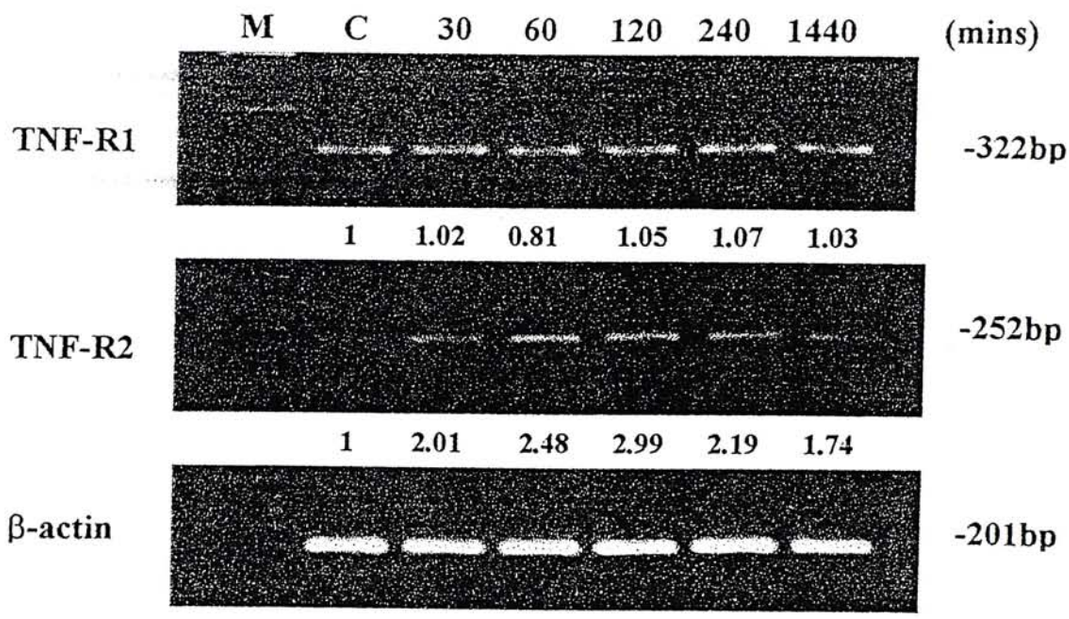

Fig. 26 Time course of procaterol treatment on TNF-R1, -R2 and β-actin mRNA in C6 cells. C6 cells were treated with 10 nM procaterol from 30 minutes to 24 hours, and the total RNA was extracted and followed by RT-PCR as described in Methods. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

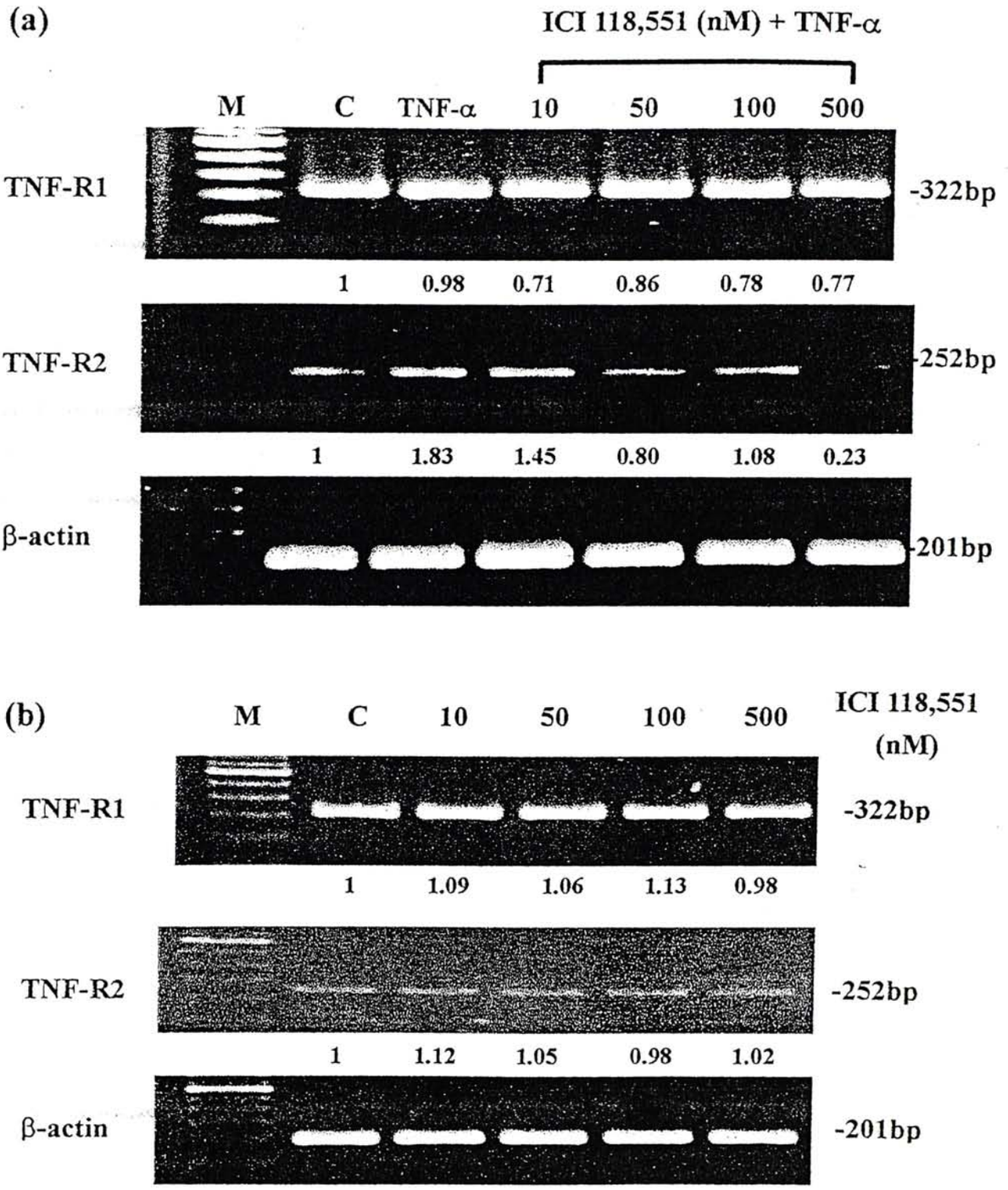

Fig. 27 Effect of various concentrations of ICI 118,551 on the levels of TNF-R1, -R2 and β-actin mRNA in TNF-α-treated C6 cells. (a) C6 Cells were treated with 100 U/ml TNF-α, or pre-treated with 10 nM to 0.5 μM ICI 118,551 for 2 hours, before the treatment of TNF-α for another 2 hours. (b) C6 Cells were treated with 10 nM to 1 μM ICI 118,551 for 4 hours. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

binding and NF-κB expression was also investigated.

3.4.1 Effect of TNF-α on NF-κB (p50) mRNA expression in C6 cells

In this study, C6 cells were treated with 100 U/ml TNF-α for different time periods: 5 minutes to 48 hours. The mRNA level of NF-κB was semi-quantified by RT-PCR. The NF-κB mRNA expression was enhanced in a time-dependent manner in cells treated with TNF-α, and the maximum induction (about 7.5 folds) was observed at around 2 to 4 hours then declined (Fig. 28). This indicates that NF-κB can be induced by TNF-α, and it maybe that this gene participates in the TNF-α-induced proliferation of C6 cells.

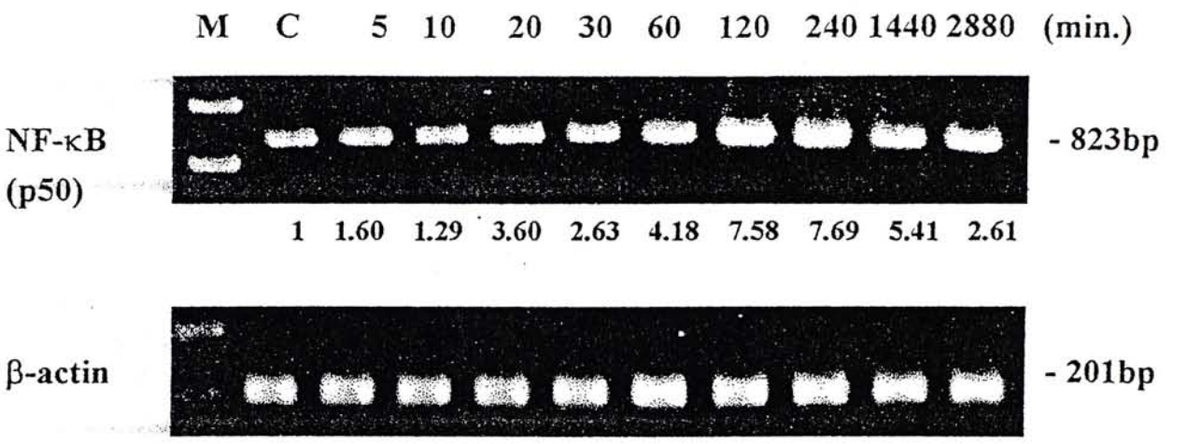

Fig. 28 Time course of the effect of TNF-α on the levels of NF-κB/p50 subunit and β-actin mRNA in C6 cells. C6 Cells were treated with 100 U/ml TNF-α from 5 minutes to 48 hours, and the total RNA was extracted and semi-quantified by RT-PCR as described in the Methods. After 25 cycles of PCR, a 5-μL aliquot of the PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide then photographed. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

3.4.2 Effects of β-agonist and antagonist on NF-κB (p50) mRNA expression in C6 cells

In our laboratory, we have found that both TNF-α and β-agonist can induce the C6 cell proliferation (Liu, 1996; Lung, 1999) and that TNF-α-induced β-AR expression (Fig. 10), this suggests that β-adrenergic mechanism is also closely related to C6 cell proliferation. So, it is of interest to investigate whether there is a relationship between β-adrenergic mechanism and NF-κB expression in C6 cells. In this study, different dosages (0.01, 0.1, 1 and 10 μM) of an β-agonist, isoproterenol, were used to study its effect on NF-κB mRNA expression in C6 cells, and the mRNA levels were semi-quantified by RT-PCR. The expression of NF-κB mRNA was enhanced by about 2 folds when exposed to low concentrations (0.01 to 0.1 μM) of isoproterenol, and the induction were reduced when the concentrations of the β-agonist were further increased (Fig. 29). Moreover, the β-antagonist, propranolol, suppressed the TNF-α-induced NF-κB at all doses of propranolol tested, by about 56% with 0.05 μM of propranolol, and almost complete inhibition (back to the control level) with 25 μM (Fig. 29a). Propranolol alone, at all concentrations tested, did not have any significant effects on NF-κB mRNA expression (Fig. 29b). This suggests that β-adrenergic mechanism regulates the expression of this nuclear factor and that is

involved in mediating the TNF-α-induced NF-κB expression in C6 cells.

3.4.3 Effects of PMA and Ro31 on the levels of NF-κB mRNA in C6 cells

Results described before (Figs. 5-8) revealed that TNF-α exerted its action in C6 cells by activating PKC, we, therefore, examined the effects of PMA and Ro31 on NF-κB expression. In this study, cells were exposed to 0.0162, 0.162, 1.62, 16.2 and 162 nM of PMA, and its effect on NF-κB mRNA expression was examined. As shown in Fig. 30, 0.0162 nM of PMA, the lowest concentration tested, had the most pronounced induction effect (3.2 folds) on NF-κB mRNA expression. As the concentrations of PMA increased, the mRNA expression declined, and at 162 nM an inhibition was observed. This suggests that the activation of PKC induced NF-κB mRNA expression.

To ensure the effect of PMA is specific, the effect of various doses of Ro31 on the levels of TNF-α-induced-NF-κB mRNA expression was studied (Fig. 31). C6 Cells were treated with 10, 50, 100, 200 and 500 nM of Ro31 for 2 hours before the exposure to TNF-α (100 U/ml) for 2 hours, and the NF-κB mRNA levels semi-quantified by RT-PCR. Ro31 reduced the TNF-α-induced NF-κB expression (Fig. 31a). This inhibitory effect of the PKC inhibitor was dose-dependent, a clear

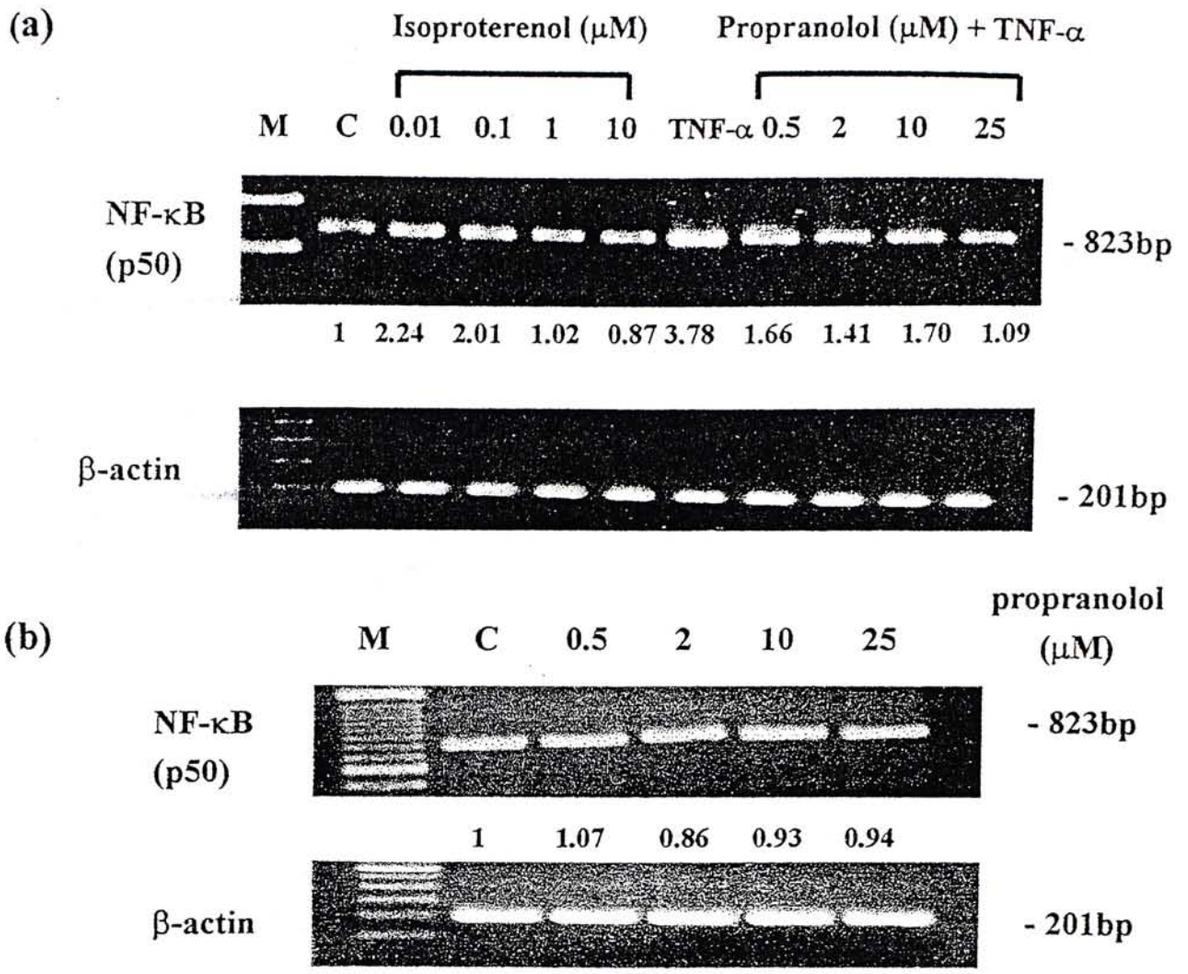

Fig. 29 Effects of isoproterenol, propranolol and TNF-α on the levels of NF-κB/p50 subunit and β-actin mRNA in C6 cells. (a) C6 Cells were treated with 0.01 to 10 μM isoproterenol, 100 U/ml TNF-α for 2 hours, or pre-treated with propranolol (0.5 to 25 μM) for 2 hours before the addition of 100 U/ml TNF-α for another 2 hours. The untreated cells (C) served as the control. The sizes, in bp, of the PCR products were indicated on the right. The DNA markers (M) were also run on the same gel. Other details were as described in Fig. 28. Data presented are representations of three separate experiments with similar results.

suppressive effect (28%) was observed with 10 nM of Ro31 and higher inhibitory were observed with higher concentrations of Ro31(Fig. 31a). Moreover, Ro31 alone did not affect the expression of NF-κB at all the concentrations tested (Fig. 31b). These results suggest that PKC is involved in the TNF-α-induced NF-κB gene expression in C6 cells.

3.5 Effects of TNF-α on the expression of manganese superoxide dismutase (MnSOD) in C6 glioma cells

As discussed in Section 1.11, an overexpression of MnSOD has been shown to prevent neuronal cell death by its suppression of peroxynitrite production and lipid peroxidation (Keller *et al.*, 1998). Moreover, TNF-α receptor activation results in activation of the NF-κB, which may serve as an antiapoptotic role via the induction of target genes MnSOD (Mattson, 1997; Cai & Jones, 1998). These suggest that TNF-α action, activation of NF-κB, MnSOD expression and anti-apoptosis are closely associated.

As both TNF-α and isoproterenol induce C6 cell proliferation (Liu, 1996; Lung, 1999), we therefore investigated the relationship between TNF-α, β-adrenergic mechanism and MnSOD expression in C6 cells. From the results described in

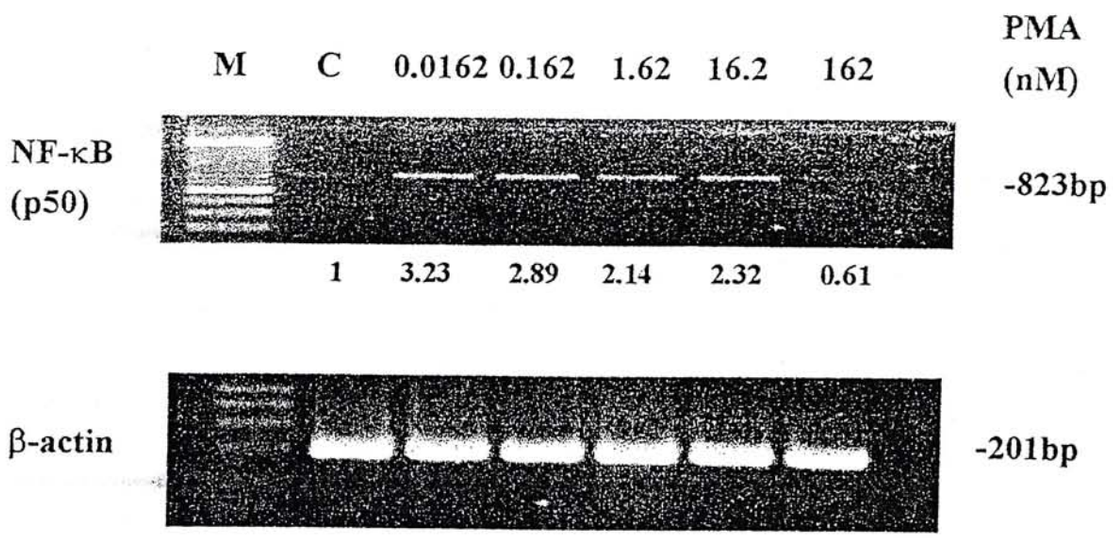

Fig. 30 Effect of various concentrations of PMA on the levels of NF-κB/p50 subunit and β-actin mRNA in C6 cells. C6 Cells were treated with PMA (0.0162 nM to 0.162 μM) for 2 hours. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

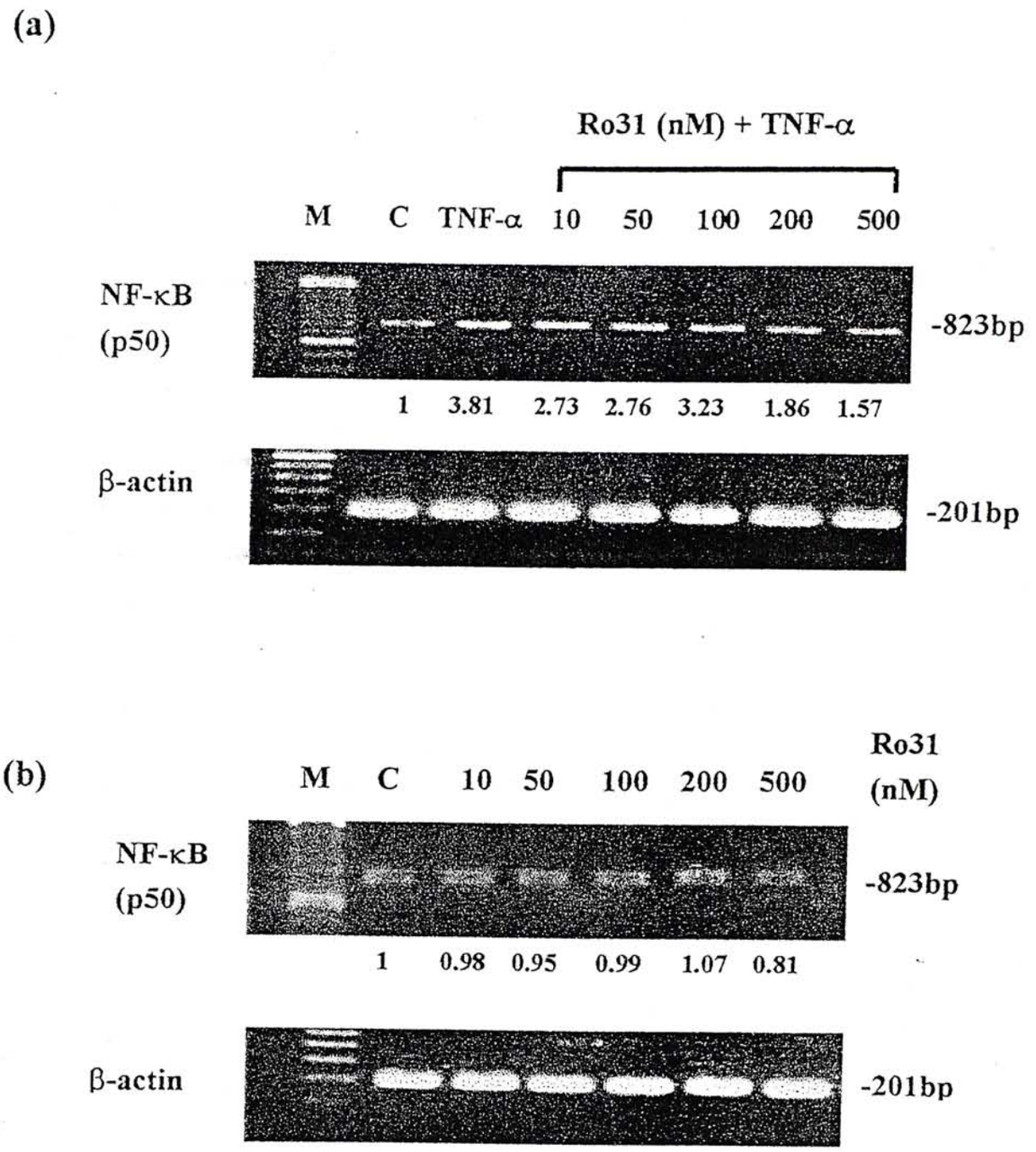

Fig. 31 Effect of various concentrations of Ro31 on the levels of NF-κB/p50 subunit and β-actin mRNA in TNF-α-treated C6 cells. (a) C6 Cells were treated with various concentrations of Ro31 for 2 hours before exposed to 100 U/ml TNF-α. (b) C6 Cells were treated with 10 nM to 500 nM Ro31 for 4 hours. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

Sections 3.3.2 and **3.3.3** that the activation of PKC and β-adrenergic mechanism resulted in the elevation of NF-κB mRNA levels in C6 cells. We also examined the signal transduction mechanism between TNF-α and MnSOD expression in these cells. Another very important reason is because NF-κB activation induces the expression of MnSOD (Mattson, 1997; Cai & Jones, 1998).

To delineate this relationhsip, the effects of various agents (to be described below) on the MnSOD gene expression in C6 cells were measured by RT-PCR technique as described in **Section 2.5**.

3.5.1 Effect of TNF-α on MnSOD and Cu-ZnSOD mRNAs expression in C6 cells

Besides MnSOD, copper-zinc superoxide dismutase (Cu-ZnSOD) is the other enzyme that can capture and reduce free radicals, and preventing the oxidative damage in a cell (Wong *et al.*, 1989). As TNF-α had been shown to have no effect on Cu-ZnSOD in kidney cells (Wong *et al.*, 1989), we therefore investigated if TNF-α also selectively induced MnSOD in C6 cells.

In this study, cells were exposed to different doses of TNF-α (50, 100, 500 U/mL) for 2 hours, and the levels of MnSOD, Cu-ZnSOD and β-actin mRNA in C6 cells measured by RT-PCR. As shown in Fig. 32, the MnSOD mRNA level was

enhanced at all concentrations of TNF-α tested, with the optimal induction (2.5 folds) at 100 U/ml TNF-α. On the other hand, no effects on Cu-ZnSOD and β-actin mRNA expression were observed with all concentrations of TNF-α tested (Fig. 32). This indicates that MnSOD is selectively induced by TNF-α in C6 cells. As the expression of Cu-ZnSOD was not affected, no further study on this enzyme was performed.

3.5.2 Effects of β-agonist and β-antagonist on MnSOD mRNA expression in C6 cells

Previous studies in our laboratory found that β-adrenergic mechanism was related to C6 cell proliferation (Liu, 1996; Lung, 1999) and that MnSOD was reported to be antiapoptotic (Mattson, 1997; Cai & Jones, 1998), it is of interest to examine the action of isoproterenol and propranolol on MnSOD expression in C6 cells. In the first study, cells were exposed to different doses (0.01, 0.1, 1 and 10 μM) of isoproterenol, and its effect on MnSOD mRNA expression were semi-quantified by RT-PCR (Fig. 33a). The level of MnSOD mRNA was induced by all concentrations of isoproterenol tested, and higher levels of induction (about 14 folds) were observed with 0.01 to 1 μM then slightly declined with 10 μM. Prior treatment with propranolol (0.5 to 25 μM) suppressed the TNF-α-induced MnSOD mRNA expression, and there was a concentration-dependence (Fig. 33a). At 25 μM, which effectively suppressed C6 cell

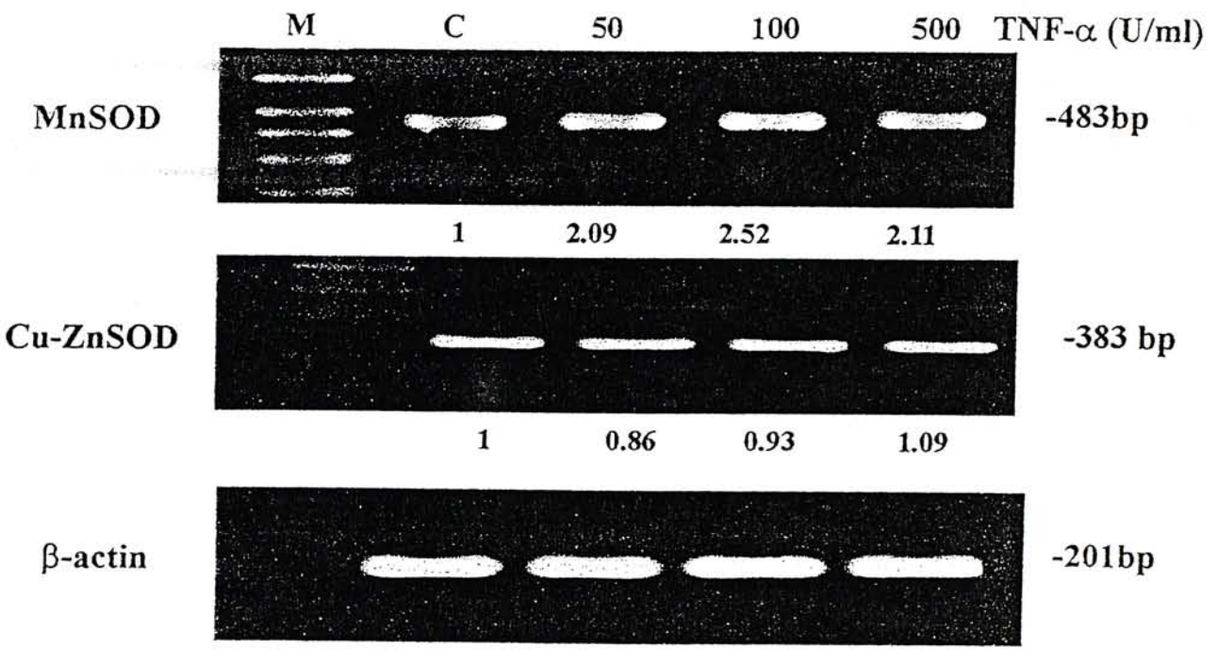

Fig. 32 Effects of various concentrations of TNF-α on the levels of MnSOD, Cu-ZnSOD and β-actin mRNA in C6 cells. C6 Cells were treated with 50 to 500 U/ml TNF-α for 2 hours, and the total RNA was extracted and semi-quantified by RT-PCR as described in the Methods. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

proliferation (Liu, 1996), the induction was about 52%. However, propranolol alone, except at high concentrations, did not have any clear effect on MnSOD mRNA expression (Fig. 33b). As TNF-α can induce the expression of β-ARs (Fig. 10), it is quite possible that an β-adrenergic mechanism is involved in mediating TNF-α-induced MnSOD expression in C6 cells. The issue whether there is a differential effect of β1- and β2-adrenergic mechanism in regulating MnSOD expression is currently under investigation.

3.5.3 Effects of PKC activator and inhibitor on the levels of MnSOD mRNA in C6 cells

Results presented earlier showed that PKC was involved in the induction of TNF-R2 and β-ARs (Figs. 5, 6, 11 and 12), so it is important to examine whether PKC also regulate MnSOD expression in C6 cells. In this study, cells were exposed to different doses (0.0162, 0.162, 1.62, 16.2 and 162 nM) of PMA, and the levels of MnSOD mRNA were semi-quantified by RT-PCR (Fig. 34). The level of MnSOD mRNA was increased by almost 1 fold with low concentrations (0.0162 and 0.162 nM) of PMA, then slightly declined or returned to the control level with higher concentrations of PMA. This suggests that activation of PKC induces MnSOD mRNA

expression in C6 cells.

Next, the dosage effects of Ro31, in the presence or absence of TNF-α, on the levels of MnSOD mRNA were studied (Fig. 35a). C6 Cells were pre-treated with 10, 50, 100, 200 and 500 nM Ro31 for 2 hours before exposed to TNF-α for 2 hours and the levels of MnSOD mRNA were semi-quantified by RT-PCR. This inhibitory effect of Ro31 was dose-dependent, the inhibition was about 35% with 10 nM Ro31 and greater suppression was observed with higher concentrations of Ro31. Moreover, Ro31 alone did not change the expression of MnSOD at all the concentrations of this inhibitor tested (Fig. 35b). These results suggest that PKC is involved in the TNF-α-induced MnSOD gene expression in C6 cells.

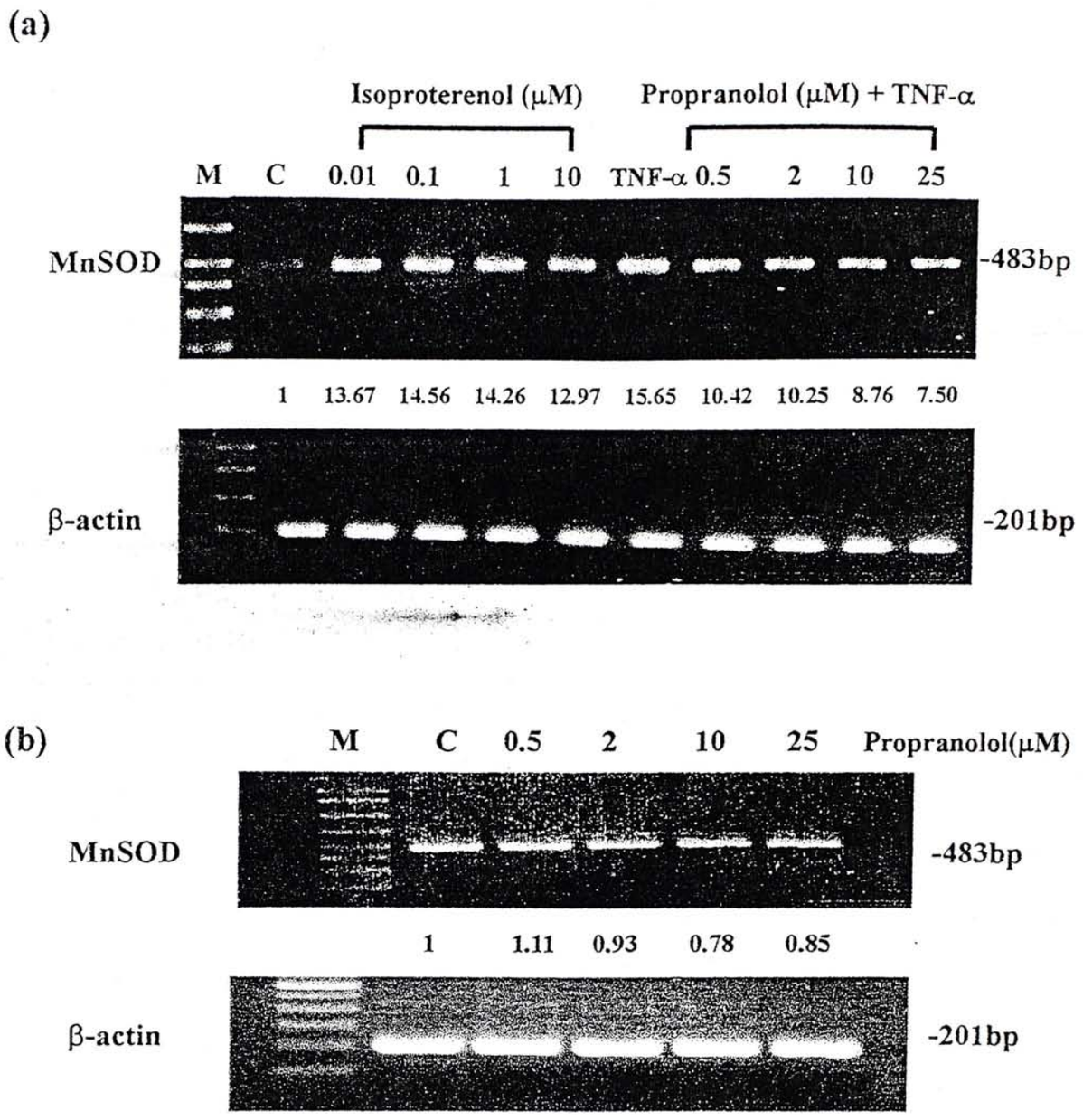

Fig. 33 Effects of various concentrations of isoproterenol and propranolol and TNF-α on the levels on MnSOD and β-actin mRNA in C6 cells. (a) C6 Cells were treated with 0.01 to 10 μM isoproterenol or 100 U/ml TNF-α for 2 hours, or pre-treated with propranolol (0.5 to 25 μM) for 2 hours before addition of 100 U/ml TNF-α for another 2 hours. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

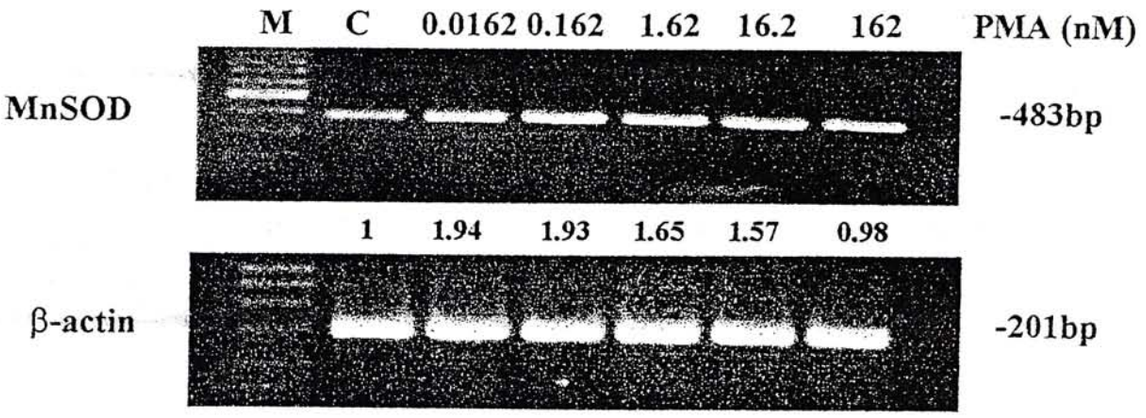

Fig. 34 Effects of various concentrations of PMA on the levels of MnSOD and β-actin mRNA in C6 cells. C6 Cells were treated with 0.0162 to 162 nM of PMA for 2 hours, and the total RNA was extracted and followed by RT-PCR as described in the Methods. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

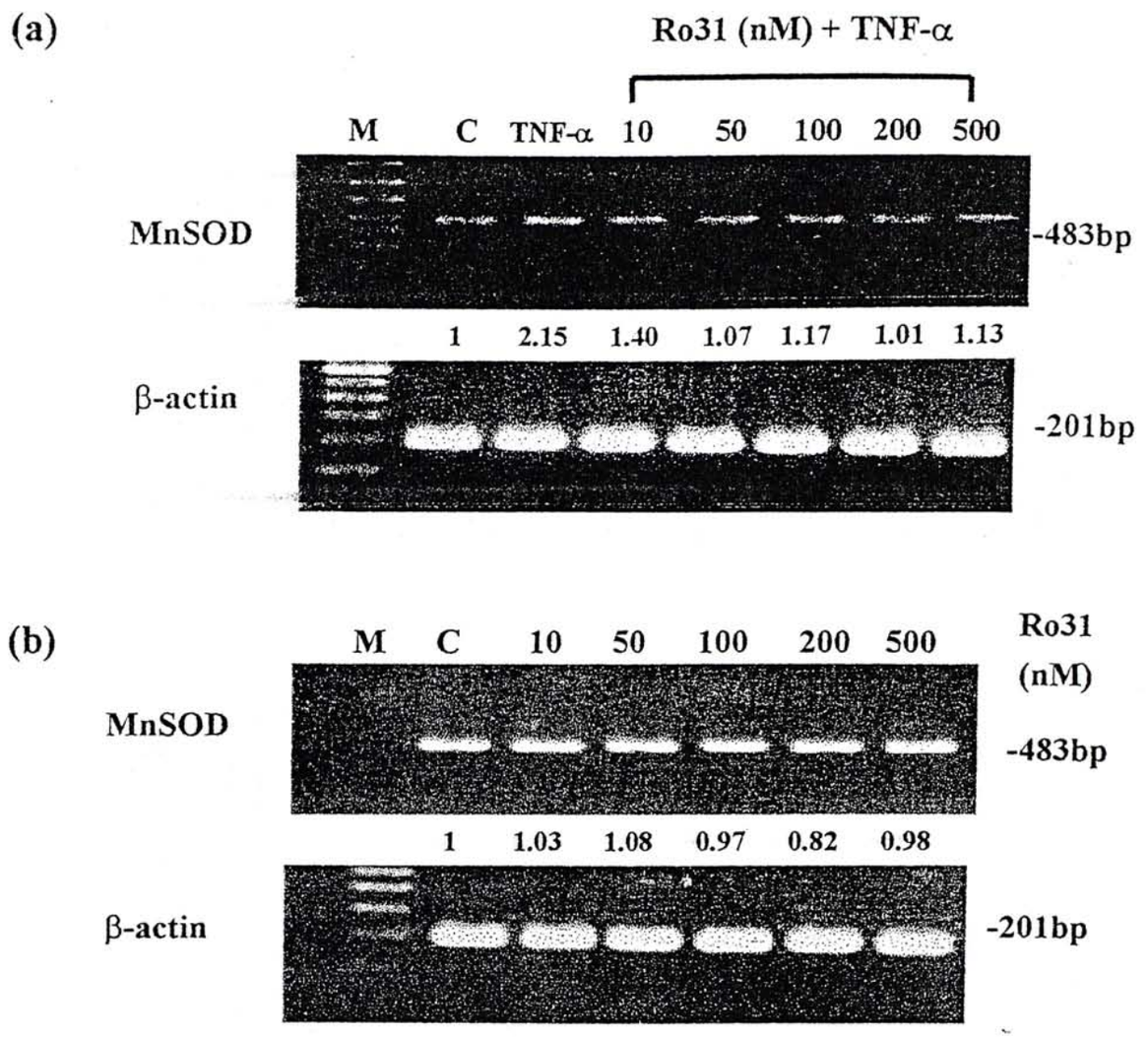

Fig. 35 Effects of various concentrations of Ro31 on the levels of MnSOD and β-actin mRNA in TNF-α-treated C6 cells. (a) C6 Cells were treated with 100 U/ml TNF-α for 2 hours, or pretreated with 10 to 500 nM Ro31 for 2 hours before the addition with TNF-α for another 2 hours. (b) C6 Cells were treated with 10 to 500 nM Ro31 for 4 hours. The untreated cells (C) served as the control. Other details were as described in Fig. 4. Data presented are representatives of three separate experiments with similar results.

4. DISCUSSION AND CONCLUSION

4.1 Effects of TNF-α on the expression of TNF-receptors (TNF-Rs) in C6 glioma cells

C6 Glioma cells were used as a model to examine the responses of astrocytes to TNF-α, as they respond to cytokines like astrocytes (Munoz-Fermandoz *et al.*, 1991; Munoz-Fermandoz & Fresno, 1993; Brodie & Goldreich, 1994). Moreover, they are easy to grow in culture and with short population doubling time (Benda *et al.*, 1968; Westermark *et al.*, 1973).

It was previously reported many cell types contained both TNF-R1 and –R2 (Ware *et al.*, 1991; Barbara *et al.*, 1994; Dopp *et al.*, 1998). In our laboratory, we have found that C6 cells (Huang *et al.*, 1998; Lung, 1999; To, 1999) and primary cultured astrocytes (Lung *et al.*, unpublished results) also contain both receptor subtypes. As both the levels of TNF-R1 mRNA and protein were higher in the untreated C6 cells (Fig. 7), it is quite possible that this receptor subtype is the predominant subtype in normal (untreated) C6 cells. This finding is in agreement with the report that TNF-R1 is the predominant receptor subtype in epithelial cells (Ware *et al.*, 1991) and primary astrocytes (Dopp *et al.*, 1997). The lower density of TNF-R2 on the surface of a cell does not, however, limit the interaction between TNF-α and TNF-R2, as this receptor

subtype has higher affinity for this cytokine (Bluethmann, 1998). Moreover, this subtype can be selectively induced (Fig. 4).

Results from the present study showed that TNF-α selectively induced TNF-R2 mRNA (Fig. 4), as well as its protein (Fig. 8) expression in a time- and dose-dependent manner (Fig. 4; Lung, 1999). As TNF-α induces C6 cell proliferation (Liu, 1996; Lung, 1999; To, 1999), the present findings suggest that this selective induction of TNF-R2 is related to C6 cell proliferation. This proposed is in line with the thought of Tartaglia and his colleagues (Tartaglia *et al.*, 1991; Tartaglia & Goeddel, 1992; Tartaglia *et al.*, 1993) who based on their studies with the immune system cells suggested that TNF-R1 mediates cytotoxicity, which TNF-R2 enhances mitogenic effect. Moreover, the cytotoxic action of TNF-R1 was also observed with Hep-2 cells (a cell line derived from human carcinoma of the larynx), U937 (a human monoblastoid cell line) (Barbara *et al.*, 1994) and endothelial cells (Stoelcker *et al.*, 2000).

Indeed, To (1999) in our laboratory showed that TNF-R2 antibody, but not TNF-R1 antibody, inhibited the proliferative effect of TNF-α in C6 cells. Similar finding has been reported in cultured microglia (Dopp *et al.*, 1997). The differential roles of these receptor subtypes in response to TNF-α has been clearly demonstrated by Shen *et al.* (1997) in their study with SH-SY 5Y cells (a human neuronal cell line),

where they found that treatment of these cells with antisense oligonucleotides for TNF-R2, but not for TNF-R1, significantly increased cell death due to TNF-α treatment. This suggests that TNF-α and TNF-R2 play a protective role by forming glial scar following neuronal injury as TNF-α had been shown to be elevated in brain injury (Fan *et al.*, 1996; Kamei *et al.*, 2000). The importance of TNF-R2 in cell proliferation was clearly demonstrated by To (1999) in our laboratory by using selective TNF-R antibodies. In addition to the selective induction of TNF-R2 in nerve tissues, TNF-R2 had been found to be specifically increased in brain microvessels in mice exposed to experimental cerebral malaria (CM) (Lucas *et al.*, 1997). These authors also found that mice deficient in TNF-R2 were not significantly protected from experimental CM.

In short, our findings, together with previous reports (cited above), strongly suggest that the selective induction of TNF-R2 by TNF-α is closely related astrocyte proliferation, astrogliosis, scar formation and the outcome of brain injury. Moreover, this selective induction may offer a protection in certain neurological conditions.

As PKC is a common second messenger in many cell types, and it has been found to be the signal transduction mediating TNF-α actions in a human erythroblastoid leukemic cell line, K562 (Zhang *et al.*, 1994), we investigated the role of PKC in the TNF-α-induced proliferation in C6 cells. We found that PMA induced

both the TNF-R2 mRNA and protein levels in C6 cells (Figs. 5 & 7). Both the TNF-α-induced TNF-R2 mRNA and protein were suppressed by Ro31, a selective inhibitor of PKC in C6 cells (Tsang *et al.*, 1997) (Figs. 6 & 8). This suggests PKC is involved in TNF-α-induced TNF-R2 expression. The roles played by different PKC isoenzymes in mediating this action of TNF-α in C6 cells had been studied in our laboratory. Lung (1999) demonstrated that the increase in PKC-ζ following TNF-α treatment was most rapid among the PKC isoforms investigated. Moreover, To (1999) in her study of TNF-α and intracellular Ca^{2+} movement in C6 cells found that TNF-α induced the elevation of intracellular free Ca^{2+} levels in C6 cells, and that A23187, a Ca^{2+} ionophore, induced the expression of TNF-R2 and PKC-γ, a Ca^{2+}-dependent isoform (Das *et al.*, 1998). A similar up-regulation of γ isoform message has also been reported in gerbil hippocampus after transient cerebral ischemia (Zablocka *et al.*, 1998). Thus, it is likely that both PKC-γ and -ζ play more important roles in the enhanced expression of TNF-R2 in C6 cells.

Besides PKC, the possible involvement of PKA in the TNF-R2 expression was also studied. Our study showed that dbcAMP, an well-established activator of PKA (Messens & Slegers, 1992), did not elevate either TNF-R1 or –R2 mRNA levels in C6 cells (Fig. 9). Thus, it is likely that the PKA was not involved in the TNF-α-induced TNF-R2 gene expression in C6 glioma cells. In addition, Lung (1999) in our

laboratory showed that tyrosine kinase (TK) and nitric oxide (NO) inhibitors were unable to suppress the TNF-α-induced effect on TNF-R2 expression. At present, we believe that PKC is a major messenger involved in the TNF-α-induced TNF-R2 expression. As SMase has been shown to mediate TNF-α action (Barbara ***et al.***, 1996), the involvement of this and other signaling pathways in C6 cells cannot be excluded.

4.2 Effects of TNF-α on the expression of β1- and β2-adrenergic receptors (β1- and β2-ARs) in C6 glioma cells

Besides TNF-α, β-adrenergic mechanism has found to be related to astrocyte proliferation, astrogliosis and glial scar formation observed following brain injury (Griffith & Sutin, 1996). Recently, Lung (1999) has demonstrated that isoproterenol, activated C6 cell proliferation, while propranolol inhibited TNF-α-induced cell proliferation. This suggests that TNF-α and β-adrenergic mechanism are closely related to proliferation in C6 cells. Indeed, we found that the addition of TNF-α to C6 cells resulted in an increase in the β1- and β2-AR mRNA levels in a time- (Fig. 10) and dose-dependent manner (Lung, 1999). Both β-AR mRNA levels were enhanced in 5 minutes and that β1- and β2-AR reached their optima after 30 and 60 minutes, respectively, following exposure to TNF-α. It seems that the response of β1-AR

expression (5.4 folds at 5 mins.) to TNF-α was faster than that of β2-AR (Fig. 10). Both β1- and β2-AR mRNAs were expressed in C6 cells, this suggests that both β-ARs co-exist in C6 cells, and this is in agreement with the other studies with C6 cells (Hough & Chuang, 1990; Zhong & Minneman, 1993). Furthermore, the decrease in both β-AR mRNA levels after 24 hours maybe due to the prolonged, but negative action of TNF-α. Although we had not examined whether this induction is also mediated through the activation of TNF-R2, based on our findings that both TNF-α (Fig. 4) and isoproterenol (Fig. 16) induced the expression of TNF-R2, and that both agents induced cell proliferation (Lung, 1999), it is likely the result observed is mediated through TNF-R2. (We are currently investigating this issue using specific TNF-R1 and –R2 antibodies.)

As TNF-α induced the expression of both β-AR mRNA in C6 cells and that the induction of TNF-R2 was mediated by PKC (Figs. 5-8), we studied if PKC is the mediator in the TNF-α-induced β-AR. The treatment of PMA resulted in an increase in the expression of both β1- and β2-AR mRNA as well as the protein levels in C6 cells (Figs. 5 and 7), and maximum expression of both proteins was observed at 48 hours (Fig. 7). Furthermore, TNF-α-induced β1- and β2-AR mRNA and protein expressions were suppressed by Ro31, and the sensitivities of both β-ARs were about the same (Figs. 6 and 8). These suggest that the TNF-α increased both β-ARs via the

PKC pathway. The issue whether different PKC isoforms play differential roles in the induction of β-ARs was not examined in this study.

As mentioned before, besides PKC, PKA was also interested to be tested. We have found that the PKA activator, dbcAMP, could not induce both β-ARs mRNAs (Fig. 9). This suggests that the activation of β-AR expression was not mediated through PKA, but via PKC. Moreover, Lung (1999) in our laboratory showed that TK inhibitors could not attenuate the TNF-α-induced β-AR expression in C6 cells. Thus, it is suggested that the elevation of β-ARs was not mediated via a TK pathway. It is likely that the the PKC signaling pathway is one of the more important pathways in this induction process.

4.3 Relationship between TNF-α and β-adrenergic mechansim in C6 cells

Our results showed that β-AR expression was induced by TNF-α (Fig. 10), and Lung (1999) has also found that the TNF-α-induced C6 cell proliferation was mediated through β-adrenergic mechanism, we attempted to see if β-adrenergic mechanism regulates endogenous TNF-α and TNF-R2 mRNA expression.

Results in the present study showed isoproterenol could induce both endogenous TNF-α (maximum 10 folds) and TNF-R2 mRNA (maximum 5.6 folds)

expression in a dose-dependent manner (Figs. 16 and 17). Furthermore, propranolol suppressed the TNF-α-induced endogenous TNF-α and TNF-R2 mRNA in a dose-dependent manner (Figs. 16 and 17). These suggest that β-adrenergic mechanism regulates endogenous TNF-α, and TNF-R2 gene expression in C6 cells. Since there are at least 2 β-AR subtypes in the brain (Imura *et al.*, 1999) and C6 cells (Hough & Chuang, 1990; Zhong & Minneman, 1993), we investigated which receptor subtype plays a more important role in inducing the expression of TNF-R2 and endogenous TNF-α in C6 cells. Our study showed that both β1-agonist, dobutamine, and β2-agonist, procaterol, could induce endogenous TNF-α mRNA expression in a dose-dependent manner (Figs. 18 and 23), and also selectively enhanced the TNF-R2 mRNA expression in a dose- and time-dependent manner (Figs. 20, 21, 25 and 26). This indicated that activation of both β1- and β2-AR could result in the induction of endogenous TNF-α and TNF-R2 gene expressions in C6 cells. Supporting this notion is our finding that the TNF-α-induced endogenous TNF-α and TNF-R2 mRNA expressions were inhibited by selective β1-antagonist, atenolol and β2-antagonist, ICI 118,551 (Figs. 19, 22, 24 and 27). Thus, this suggested that both β1- and β2-ARs could regulate the expression of endogenous TNF-α and TNF-R2 in C6 cells.

In comparing the results obtained with selective β1- and β2-agonists and -antagonists (Figs. 20-22, 25-27), we found that β2-agonist was more effective (Fig. 25)

in inducing the expression of TNF-R2 expression, while β1-antagonist caused a relative large inhibition (>3-fold) of TNF-α-induced TNF-R2 expression (Fig. 22). We tentatively concluded that both β-ARs are important in the regulation of TNF-α and TNF-R2 expression in C6 cells. This thought is agreement with the report that both β-ARs were expressed in C6 cells (Hough & Chuang, 1990; Zhong & Minneman, 1993), that norepinephrine induced the IL-6 production in astrocytes (Norris & Benvensite, 1993) and that isoproterenol increased IL-10 production in mouse peritoneal macrophages (Suberville *et al.*, 1996). Moreover, Guirao *et al.* (1997) found that catecholamines increased monocyte TNF-R2, but not TNF-R1 expression, and inhibited that of TNF-α via β2-adrenergic activation. Moreover, Stevern *et al.* (1992) found that preincubation of THP-1 cells with isoproterenol before LPS stimulation resulted in increased TNF-α production. Also, Murray *et al.* (2000) reported that isoproterenol significantly increased TNF-α mRNA and protein expression in myocardium. However, Nakamura *et al.* (1999) demonstrated that the stimulatory effect of LPS on TNF-α transcription was suppressed by isoproterenol in the rat renal resident macrophage cells. This report though contradicts our findings, nevertheless supports the notion there interactions exist between TNF-α and β-adrenergic mechanism. These contradictions maybe due to different cell lines used.

4.4 Effects of TNF-α on the expression of a transcriptional factor Nuclear Factor Kappa B (NF-κB) in C6 glioma cells

Following the investigation on the second messenger, it would be natural to examine the response of transcription factors, in this study we concentrated on NF-κB, in C6 glioma cells. This factor was chosen as it had been reported that TNF-α activation resulted in the activation of NF-κB, which may in turn induce some target genes, for example, MnSOD and/or calbindin (Sullivan *et al.*, 1999). Moreover, NF-κB has been shown to be activated by some cytokines and neurotrophic factors and in response to various cell stressors (Mattson *et al.*, 2000). More related to our study are the reports which that showed NF-κB activity was increased rapidly in the cortex in traumatic brain injured rats (Yang *et al.*, 1995; Mattson *et al.*, 2000). The expression of the p50 subunit was chosen for this study because after the NF-κB complex was activated, its inhibitory subunit, IκB dissociated from the complex, then the p50-p65 dimer translocates to the nucleus where the p50 subunit bound to the sequence in genes responsive to NF-κB (Tan *et al.*, 1994; Mattson *et al.*, 2000).

Present study showed that the expression of NF-κB/p50 mRNA in C6 cells was much enhanced by the treatment of TNF-α (Fig. 28), though it was already found in untreated C6 cells. This is in good agreement with a recent study that NF-κB is

expressed in C6 glioma cells (Lim *et al.*, 2000). Moreover, as the NF-κB activity was increased after brain injury (Yang *et al.*, 1995; Mattson *et al.*, 2000), thus, it is possible that NF-κB is induced by the elevated TNF-α after brain injury (Fan *et al.*, 1996). This induction could in turn induce other target genes, for example, the antioxidant enzyme MnSOD, the calcium-binding protein calbindin D28k, and members of the inhibitor of apoptosis family of proteins (Mattson *et al.*, 2000) in order to enhance the proliferative effect of TNF-α. Based on our study, it is likely these effects are mediated through TNF-R2 and β-AR induction as they were selectively increased by TNF-α (Figs. 4 and 10).

As β-ARs could regulate astrogliosis and glial scar formation following neuronal injury (Griffith & Sutin, 1996), so, we also investigated if the TNF-α-induced NF-κB/p50 mRNA expression was mediated via β-adrenergic mechanism. This factor is related astrocyte proliferation (Kirillova *et al.*, 1999; Sullivan *et al.*, 1999; Mattson *et al.*, 2000). In our study, we found that the NF-κB/p50 mRNA was enhanced with the treatment of isoproterenol in a dose-dependent manner (Fig. 29), while propranolol suppressed the TNF-α-induced NF-κB/p50 mRNA expression in C6 cells (Fig. 29). As a result, it is suggested that the TNF-α-induced NF-κB expression was mediated through an β-adrenergic system. As the stimulation with TNF-α (>7 folds, after 2-4 hours exposure) (Fig. 28) was greater than that with

isoproterenol (Fig. 29), an alternative pathway unrelated by β-adrenergic mechanism cannot be excluded.

Though TNF-α could induce NF-κB mRNA in C6 cells, the second messenger involved was still unknown. We demonstrated that PMA could enhance (~ 3 folds) the mRNA expression of NF-κB (Fig. 30). The decrease in NF-κB mRNA level in the treatment of more concentrated PMA maybe because of a down-regulation effect. This indicated that the activation of PKC could induce NF-κB mRNA expression. Furthermore, TNF-α-induced NF-κB mRNA expression was inhibited by Ro31 (Fig. 31), while there was no significant changes in NF-κB mRNA expression when treated with Ro31 alone (Fig. 31b). So, this suggested that PKC might be the mediator in the TNF-α-induced NF-κB expression in C6 cells. Thus, it is likely that the TNF-α-induced NF-κB mRNA expression maybe mediated via a PKC and β-adrenergic mechanism, but not a PKA pathway.

4.5 Effects of TNF-α on the expression of manganese superoxide dismutase (MnSOD) in C6 glioma cells

Evidence is mounting that the pivotal step in cell death is mitochondrial oxidative stress and/or dysfunction, with recent findings that apoptotic stimuli cause

increased accumulation of mitochondrial reactive oxygen species (Sullivan *et al.*, 1999). These studies also indicate that a reduction in antioxidant production would act to increase the susceptibility of neurons to insult. Superoxide accumulation is prevented by its conversion to hydrogen peroxide, a process catalyzed by the superoxide dismutases Cu/ZnSOD and MnSOD; Cu/ZnSOD is a cytoplasmic enzyme, whereas MnSOD is localized in mitochondria (Weisiger & Fridovich, 1973). Wong *et al.* (1989) have shown that treatment of cells from a kidney line with TNF-α induces the expression of mRNA for MnSOD but not for other antioxidant enzymes, such as Cu-ZnSOD, catalase, glutathione peroxidase and glutathione transferase. Interestingly, by using specific agonists and blocking antibodies to either TNF-R1 or –R2, Smith *et al.* (1994) have shown that TNF-α-mediated induction of MnSOD in human melanoma cells is via TNF-R1, but not via TNF-R2. As C6 glioma cells express both TNF-Rs, and TNF-α in C6 cells has a proliferative effect rather than cytotoxic effect, we were interested if TNF-α can induce MnSOD in C6 cells that in turn reduces apoptosis and possibly exerts its proliferative effect.

Our data suggest that TNF-α could enhance the mRNA level of MnSOD, but not of Cu-ZnSOD, in C6 cells (Fig. 32). This shows the induction of MnSOD was specific, and this is also in agreement with the report that treatment with TNF-α can induce MnSOD, but not Cu-ZnSOD, in kidney cells (Wong *et al.*, 1989). The TNF-R

subtype responsible for the MnSOD in C6 cells was not studied in this study. Although TNF-R2 was selectively induced (Figs. 4 and 8), it is still unclear if this induction does not involve TNF-R1. This is of particular importance in view of Smith *et al.* (1994) reported that TNF-R1 mediated the induction of MnSOD in human melanoma cells.

We have also found that β-adrenergic mechanism was also involved in the TNF-α-induced MnSOD expression, as isoproterenol greatly enhanced MnSOD expression, while propranolol suppressed the induction by TNF-α (Fig. 33). Thus, the induction of this enzyme in C6 cells is mediated through an β-adrenergic mechanism. In our study whether PKC was the signaling pathway on MnSOD expression in C6 cells was examined, we found that PMA increased MnSOD mRNA expression (Fig. 34) and that the TNF-α-induced MnSOD expression was prevented by Ro31, a potent PKC inhibitor (Fig. 35). This is in good agreement with a previous study with A549 cells (a human lung adrenocarcinoma cell line) showing that PMA stimulated MnSOD expression (Das *et al.*, 1998). Moreover, Kobayashi *et al.* (1997) showed that PKC inhibitors augmented TNF-α-induced apoptosis in human embryomic lung fibroblast cells by inhibiting cellular resistance factors, including MnSOD.

From the above discussion, it is clear that TNF-α induced MnSOD expression, likely via an β-adrenergic mechanism and a PKC pathway, in C6 cells. Since TNF-α

can induce both β1- and β2-AR expression in C6 cells (Fig. 10) and that both β-ARs are found in these cells (Hough & Chuang, 1990; Zhong & Minneman, 1993), it is likely both receptor subtypes are involved. The PKC isoform involved in this induction has not been investigated in the present study. In view of recent findings in our laboratory showing that PKC-ζ was rapidly induced upon the addition of TNF-α (Lung, 1999) and that PKC-γ expression was increased by A23187, a Ca^{2+} ionophore, it is tentatively suggested these two PKC isoforms may play more important role(s) in this induction process.

4.6 Possible sources of β-agonists

In the present study, we found that TNF-α induced β1- and β2-AR expression and that both receptor subtypes played a regulatory role in TNF-α, TNF-R2, NF-κB and MnSOD expression. One of the interesting points worthy of discussion is the source(s) of the β-agonist in our study. Since serum was one of the ingredients added in our cultures **(Section 2.1.2.1 and 2.1.2.2)**, and that norepinephrine (NE) and epinephrine (E) are known to be present in the serum (Dibner and Insel, 1981), it is likely that one of the possible sources of β-agonist is from the serum used. As the concentrations of NE and E in the serum are likely to be low, the possibility of other

β-agonist-like substances cannot be excluded.

4.7 Conclusions

According to our findings, a hypothesis of the relationship among the TNF-α-mediated events in C6 glioma cells can be proposed as depicted in Fig. 47. Previously, we found that C6 cell proliferation was stimulated by TNF-α (Liu, 1996; Lung, 1999). As To (1999) found that anti-TNF-R2 antibody, but not anti-TNF-R1 antibody, blocked the proliferation effect of TNF-α, thus, the proliferative effect of TNF-α is likely through the selective activation of TNF-R2 in C6 cells. After the binding of TNF-α to TNF-R2, the expression of β1- and β2-AR were induced, and this is via the PKC signaling pathway. Among the PKC isoenzymes, we believe PKC-ζ and -γ may play more important role(s) in this process. The reasons are: (1) PKC-ζ was rapidly elevated upon the addition of TNF-α (Lung, 1999); and (2) TNF-α increased the intracellular free Ca^{2+} level within seconds as demonstrated by confocal microscopy, following the addition of TNF-α, and that A23187, a Ca^{2+} ionophore, greatly and rapidly induced the expression PKC-γ (To, 1999). However, the exact mechanism would require further results with selective PKC isoform inhibitors (Das *et al.*, 1998). Activation of both β-ARs are important in the up-regulation of TNF-α and TNF-R2

expression in C6 cells. And this suggests that activation of β-adrenergic mechanism can additionally induced the proliferation of C6 cells and emphasizes the importance of the β-adrenergic mechanism in this process. The activation of β-adrenergic mechansim in turn induces the transcription factor, NF-κB, and NF-κB may play an important role in C6 cell proliferation. This is supported by the report of Kirillova *et al.* (1999), in which they found that NF-κB is an essential component of the TNF-α proliferation pathway in LE6 cells (a rat liver epithelial cell line), and that blockage of NF-κB inhibits TNF-α-induced proliferation. Subsequently, the induction of this transcription factor then induced one of its target genes, MnSOD, which can remove reactive oxygen species produced after brain injury and prevent apoptosis. Although it is uncertain whether induction of MnSOD would induce C6 cell proliferation, its induction is closely related to antiapoptosis has been clearly demonstrated (Kirillova *et al.*, 1999; Mattson *et al.*, 2000).

One of the major aims of this study is to characterize the signaling pathway mediating the TNF-α-induced astrocyte proliferation, using C6 cells as a model. As astrocyte proliferation may lead to astrogliosis which has been proposed to block axon regeneration and in turn affects the outcome of brain injury (Fawcett & Geller, 1998), thus a thorough understanding of the signaling pathway mediating the proliferation effect of TNF-α and other cytokines should proved to be beneficial to

the outcome and treatment of brain injury.

TNF-α has been shown to increase BBB permeability, damage to myelin and oligodendrocytes as well as astrocytic proliferation, a process which may lead to astrogliosis and glial scar formation following brain injury (Barone *et al.*, 1997). TNF-α has been found to be greatly elevated at the site of injury (Fan *et al.*, 1996; Kamei *et al.*, 2000) and that agents, such as TNF-α binding protein, HU-211 or soluble TNF-α receptor, which lower TNF-α are very effective in improving the outcome of brain injury (Shohami *et al.*, 1997). Our study with C6 cells demonstrated that TNF-α selectively induced the induction of TNF-R2 expression, and this is mainly mediated by PKC. These findings suggest the important roles of these two entities in brain injury.

TNF-R2 was found to responsible for the proliferative action of TNF-α in C6 cells (To, 1999). Interestingly, the protective effect of TNF-R2 to injured cells has been demonstrated using antisense oligonucleotides for TNF-R2 (Shen *et al.*, 1997) and genetically-deficient TNF-R2 mice (Lucas *et al.*, 1997). The finding that PKC is a messenger mediating the action of TNF-α in C6 cells is in line with observations in other cell types, such as erythroblastoid leukemia K562 cells (Zhang *et al.*, 1994). PKC has been shown to be a very common second messenger (Padma-peruma *et al.*, 1996; Zablocka *et al.*, 1998). Recent studies have shown that only PKC-γ, one of the

PKC isoforms that is calcium-dependent, could be particularly sensitive to ischemic insult in gerbil hippocampus (Zablocka *et al.*, 1998), and this isoform has been found to be induced after exposure to TNF-α in C6 cells (Lung, 1999) and to calcium ionophore, A23187 (To, 1999). These suggest PKC is an important step in controlling C6 cells as well as astrocytes proliferation and PKC-γ appears to be more closely associated to this process.

One of the important observations in the present study is the induction of β-ARs by TNF-α. This is because β-adrenergic blockade had been shown to reduce astrogliosis and improve the outcome of nerve injury (Sutin & Griffith, 1993; Hodges-Savola *et al.*, 1996). We found that both β1- and β2-agonists can induce TNF-α and TNF-R2 expressions in C6 cells, while their antagonists can suppress their TNF-α-induced expressions. The latter observation would provide a reasonable explanation for the beneficial effect of β-antagonists on brain injury (Sutin & Griffith, 1993; Hodges-Savola *et al.*, 1996) and emphasizes this is one of the important controlling points in treating brain injury.

Our study provides an explanation for the two seeming unrelated observations: elevation of TNF-α observed in brain injury and β-AR blockade improve the outcome of brain injury. More importantly, our results suggest that combined treatment with anti-TNF-α drugs/agents and β-AR antagonists is a more effective mean than either

compound alone for brain injury. The observation that TNF-α-induced NF-κB expression is interesting as its activity was found to be increased after brain injury (Yang *et al.*, 1995; Mattson *et al.*, 2000), and that this factor has been shown to be closely related to cell proliferation (Tan *et al.*, 1994; Yang *et al.*, 1995; Mattson *et al.*, 2000). Moreover, NF-κB has been shown to induce a number of genes, such as MnSOD, calcium binding protein calbindin D28k (Mattson *et al.*, 1995).

In agreement with studies with kidney cells (Wong *et al.*, 1989), TNF-α was found to induce MnSOD in C6 cells. The induction of MnSOD in cells is known to reduce apoptosis (Wong *et al.*, 1989; Kobayashi *et al.*, 1997; Das *et al.*, 1998). TNF-α in C6 cells does not induce apoptosis, and this maybe related to the induction of MnSOD, and its induction of TNF-R2, a receptor subtype reported to be responsible for proliferation (Tartaglia *et al.*, 1993).

If the present study has any direct bearings on the treatment of brain injury, our results suggest that blockade TNF-α action, inhibition of TNF-R2 and β-AR expression as well as their activities, PKC activity, as well as the reduction of NF-κB expression are strategic points worthy of serious consideration.

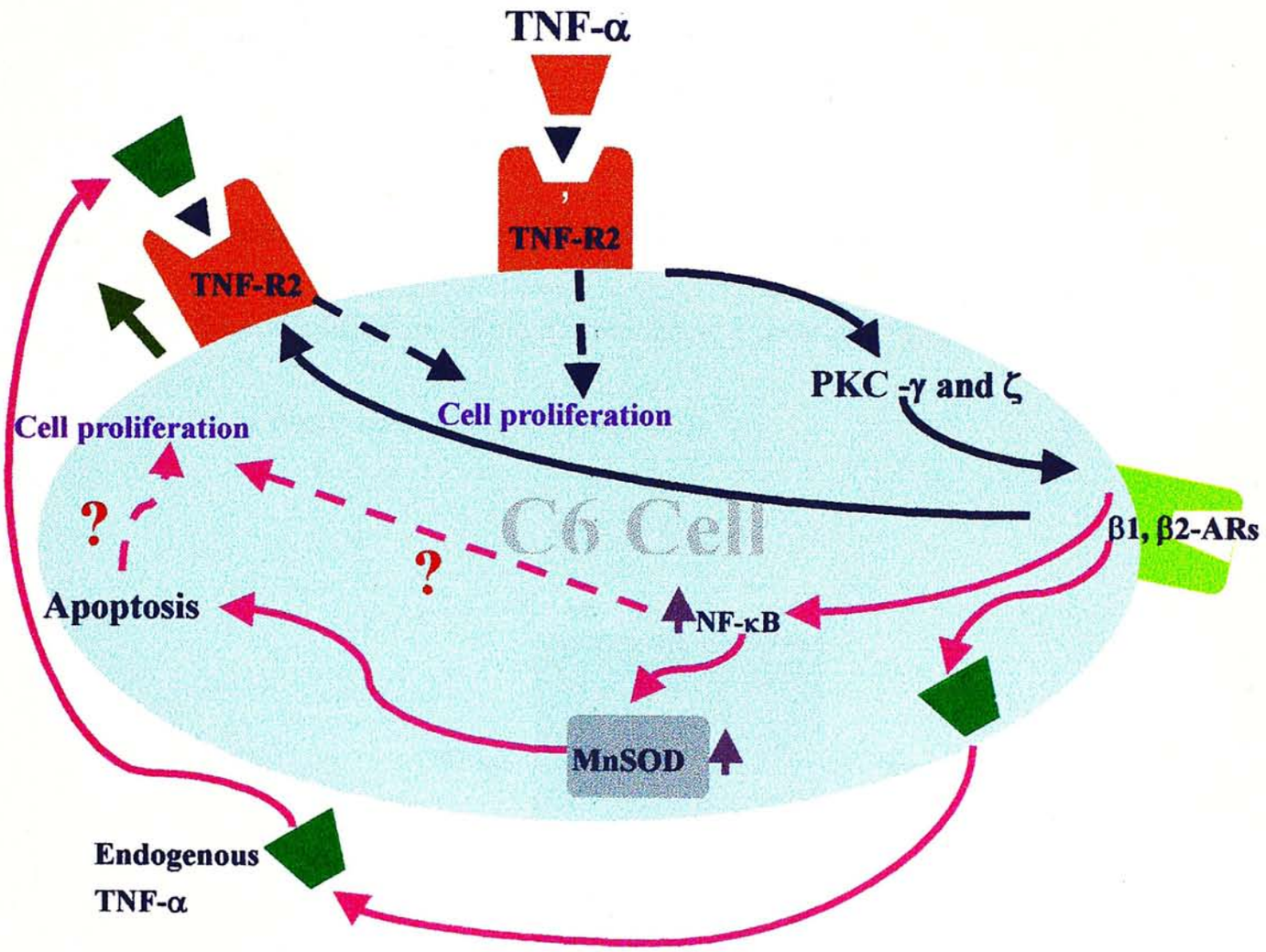

Fig. 36 Possible pathway mediating TNF-α-induced proliferation in C6 glioma cells.

Appendix A

Restriction enzyme digestion of the PCR products

The PCR bands with predicted sizes shown in this thesis were further checked by restriction enzyme (RE) digestion, except those already done by my collaborators (Huang *et al.*, 1998; Lung, 1999; To, 1999). They were found to contain the predicted restriction sites for all the amplified cDNA products. The RE digestions produced pieces with predicted sizes as shown Figs. 37 and 38, confirming that these products did result from amplification of the corresponding transcripts. Note that incomplete digestion can be observed in Cu-ZnSOD (Fig. 37).

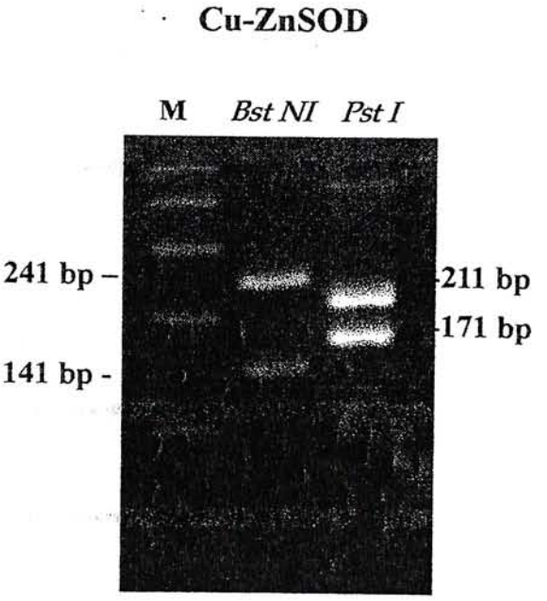

Fig. 37 Restriction enzyme digestion of amplified Cu-ZnSOD cDNA and found to contain the predicted *Bst NI* and *Pst I* restriction sites. DNA fragments with predicted restriction sizes were shown.

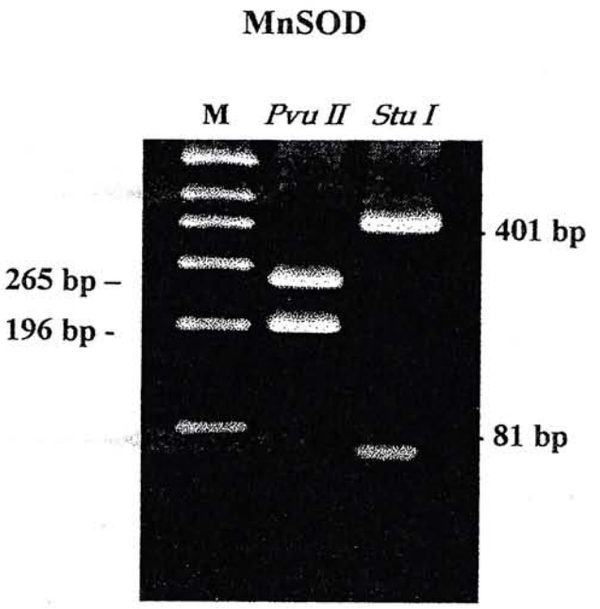

Fig. 38 Restriction enzyme digestion of amplified MnSOD cDNA and found to contain the predicted *Pvu II* and *Stu I* restriction sites. DNA fragments with predicted restriction sizes were shown.

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